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Characterization of the outer membrane proteins of *Bordetella avium*

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**Characterization of the outer membrane proteins of *Bordetella*
*avium***

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Iowa State University, 1990

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Characterization of the outer membrane proteins
of Bordetella avium

by

Randy Dean Leyh

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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Iowa State University
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1990

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PREFACE

This dissertation consists of a general introduction, a review of the literature, two separate manuscripts (SECTIONS I and II), a general summary, literature cited, and acknowledgements. The references cited in each manuscript are listed in "LITERATURE CITED" at the end of each manuscript while the references cited in the rest of the dissertation are listed in the "LITERATURE CITED" at the end of the dissertation.

GENERAL INTRODUCTION

Bordetella avium is an aerobic, nonfermentative, gram-negative rod (73, 77, 127, 130). This organism is the etiologic agent of turkey bordetellosis, an upper respiratory disease of poults which is characterized by oculonasal discharge, conjunctivitis, snicking or sneezing, reduced weight gain, and in severe cases, tracheal collapse and suffocation (9, 56, 120). Disease is frequently more severe in field outbreaks due to stress and infection by secondary pathogens (120, 132). Turkey bordetellosis is a costly disease for the turkey industry with losses resulting from reduced weight gain and increased mortality caused by secondary infections.

Like other Bordetella spp., B. avium exhibits a tropism for the ciliated epithelium of the respiratory tract. The bacteria adhere specifically to the cilia and are present on the mucosa in microcolonies (10). Colonization of the respiratory tract results in inflammation with loss of the ciliated epithelium and distortion of the mucosa and tracheal rings (9, 10). Because of the direct interaction of the bacterial surface with host cells, surface components of B. avium can play an important role in colonization of the trachea and in the subsequent immune response.

Surface appendages of B. avium include peritrichous flagella (77) and fimbriae (pili)(69). Bordetella avium possesses a poorly characterized hemagglutinin which, along with the fimbriae, might play a role in adhesion to the ciliated respiratory epithelium (14, 69).

Proteins present in the outer membrane have been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (61). Some of the outer membrane proteins (OMPs) of B. avium have been shown to induce specific antibodies during infection (11, 60). Western immunoblot analysis using tracheal washings and sera from experimentally infected turkeys has indicated that antibodies to at least eight OMPs are present locally in the trachea and systemically in the serum (60). Much of the reactivity in both sera and tracheal washings was directed to a major 21,000-molecular-weight OMP. A potential role for the surface proteins in adherence was also indicated when a B. avium mutant with reduced adherence was shown not to express certain OMPs (61).

Initial work has indicated the potential importance of the OMPs of B. avium in the pathogenesis of turkey bordetellosis. The purpose of this investigation was to further evaluate the B. avium OMPs. The OMPs were obtained by detergent extraction of total cell envelopes. The OMP profiles from numerous B. avium isolates from geographically diverse areas were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The B. avium OMPs were compared with profiles from other Bordetella spp. including numerous B. avium-like and B. bronchiseptica isolates from turkeys. The B. avium OMPs were evaluated for heat-modifiability, noncovalent association with the underlying peptidoglycan layer, and cell-surface accessibility to radioiodination. The effect of growth conditions on the expression of OMPs by B. avium was determined by varying incubation time and growth medium. Finally, the amino acid composition and the amino-terminal amino acid sequence of

the major 21,000-molecular-weight OMP of B. avium was determined. It is hoped that an improved knowledge of the OMPs of B. avium will further the understanding of their role in pathogenicity.

LITERATURE REVIEW

Classification

Turkey bordetellosis (coryza) is a highly contagious upper respiratory disease of poults characterized clinically by oculonasal discharge, sneezing, dyspnea, tracheal collapse, and reduced weight gain (9, 56, 120, 130). The disease has been reported in many countries, including Canada (42), Federal Republic of Germany (64), Australia (22), Israel (59), and the United States (130). The etiologic agent of turkey bordetellosis is a gram-negative bacterium first described as Bordetella bronchiseptica by Fillion et al. in 1967 (42). The organism was subsequently designated B. bronchiseptica-like (64) and Alcaligenes faecalis (127) and was eventually classified in 1984 as a new species, B. avium (77).

The genus Bordetella has recently been included in the family Alcaligenaceae with the genus Alcaligenes (37). Species of the genus Alcaligenes are found in soil, water, and in moist environments in hospitals, while members of the genus Bordetella colonize the respiratory epithelium of mammals and birds. The genus Bordetella contains the four species: avium, pertussis, parapertussis, and bronchiseptica (62). Bordetella avium has been isolated from at least 10 avian species including turkeys and chickens (63) but is not known to infect mammalian hosts (62, 92). Bordetella pertussis is the etiologic agent of whooping cough, a severe upper respiratory disease of children,

while B. parapertussis generally causes a milder respiratory disease in humans (62). Bordetella parapertussis has been shown to cause mild respiratory disease in lambs (33). Bordetella bronchiseptica is associated with respiratory diseases in many species including atrophic rhinitis in pigs, infectious tracheobronchitis (kennel cough) in dogs, respiratory disease in cats and laboratory animals, and infrequently, a mild whooping cough-like disease and pneumonia in humans (62).

Isolation of B. bronchiseptica is common from the upper respiratory tract of turkeys but the bacteria appear nonpathogenic when poults are experimentally infected (73, 77, 116). A second, nonvirulent organism phenotypically similar to B. avium is also routinely isolated from the turkey respiratory tract (20, 73, 116). Jackwood et al. temporarily designated this bacterium B. avium-like and suggested it may be a separate species (72).

Isolates of B. avium make up a homogeneous species phenotypically and genetically divergent from the other bordetellae (37, 77, 100). This bacterium is a strictly aerobic, nonfermentative, small rod, with an average diameter of 0.4 to 0.5 μ m and an average length of 2 μ m (77). All isolates of B. avium are motile by peritrichous flagella (77, 127). The guanine + cytosine (G+C) content of the DNA is 61.6 to 62.6 mol% (37, 77) and isolates contain various conjugative and nonconjugative plasmids which do not appear to be associated with virulence (71, 89, 131).

Bordetella avium grows best in vitro at 30°C to 35°C on common laboratory media including brain heart infusion, veal infusion,

MacConkey agar, and blood agar (12, 65, 116). Two chemically defined media for B. avium have been described (49, 89). When grown on agar for 24 h, type I B. avium colonies are small (0.5 mm), smooth, convex, and translucent with entire edges (116). This colony type was observed to be stable in vitro by Jackwood et al. (73) but in another study was shown to dissociate into a type II colony in about 10% of the isolates (65, 77). Type II colonies were larger, smooth, convex, and circular with entire edges. Recently, isolates of B. avium were observed to switch between smooth and rough colony types (70). Bacteria from both colony types had identical biochemical characteristics, however, isolates with the rough colony type were nonmotile.

Turkey Bordetellosis

Uncomplicated turkey bordetellosis is typically seen in poults 2 to 6 weeks of age and is characterized by an abrupt onset, rapid spread, high morbidity, and low mortality (120). The incubation period is 3 to 7 days with a disease course of 2 to 5 weeks (16, 56, 120). Clinical signs include snicking or sneezing, mucus accumulation at the nares, swelling of the submaxillary region, excessive lacrimal secretion, mouth breathing, dyspnea, and in severe cases, suffocation and death. Disease under field conditions is often more severe with increased mortality rates possibly due to infection by secondary pathogens (16, 27, 41, 59, 120, 143, 144) and increased stress (12, 125, 132). Though bordetellosis is mainly a problem in young turkeys, B. avium has been

associated with mild respiratory disease in turkey breeder flocks (76) and broiler flocks (21).

Gross pathologic changes in turkeys with bordetellosis include a mild to severe conjunctivitis, rhinitis, and sinusitis with mucus accumulation in the nasal cavity, sinuses, and upper trachea (9, 56, 120). Malformation of tracheal rings and softening of the tissue in the upper trachea might be present and can lead to dorsoventral flattening of the trachea and tracheal collapse with suffocation in severe cases (9, 56, 144). Gross pathologic changes in uncomplicated bordetellosis are observed only in the upper respiratory tract.

Histologic lesions in the nasal cavity and the trachea consist of fibrinopurulent rhinitis and tracheitis with loss of ciliated epithelium, bacterial colonization, epithelial hyperplasia, depletion of mucus, and a diffuse mononuclear infiltration into the subepithelial tissues (9, 56). The changes are most severe in the cranial portion of the trachea where the loss of ciliated cells can progress until as much as 95% of the normal epithelium is gone (10, 56, 119). Ciliated epithelium is replaced by nonciliated, immature cells and the mucosa is distorted into longitudinal folds (9). Bordetella avium colonizes the nasal cavity as early as one day after experimental inoculation (56). Colonization occurs in the upper trachea during the first week post-inoculation followed by a rapid spread of the organism to the lower trachea and to the primary bronchi (9, 145). The noninvasive bacteria are closely associated with basal parts of cilia and lined up with the long axis of the cilia (8, 10). Pulmonary lesions appear to be limited

to the primary bronchi (145) and include mild bronchitis with peribronchial lymphocytic hyperplasia (144).

Virulence Factors

Bacterial pathogenicity is a complex trait which is controlled by many determinants. These virulence-associated determinants or factors allow bacterial pathogens to infect a host and cause disease by permitting bacteria to colonize the host, obtain nutrients, and resist the host's immune system. Pathogens express virulence factors in vivo which are involved in each of these steps. Other determinants may have no obvious role in bacterial colonization and maintenance within the host but are important in the pathogenesis of clinical signs and lesions seen in many diseases.

Pathogenic microbes can face a variety of environments both inside and outside the host. Many bacteria have adapted to this life style by acquiring regulatory systems controlling the expression of virulence factors (44, 93). Presumably, the ability to regulate certain gene products gives the bacterium a distinct survival advantage within a particular niche. Recent interest in the coordinate regulatory system of B. pertussis and other bacterial pathogens has led to a better understanding of the molecular mechanism involved in control of expression of virulence factors.

Coordinate regulation of virulence factors has been shown to occur in B. avium in the genes encoding the dermonecrotic toxin (49) and

certain outer membrane proteins (47). The virulence regulatory system of B. avium is not well understood but it appears to be similar to the coordinate system found in the human pathogen, B. pertussis. Because of the possible relationship between the two systems, a review of the coordinate regulatory system of B. pertussis might help in understanding virulence factor regulation in B. avium.

Bordetella pertussis expresses a number of virulence factors including pertussis toxin, filamentous hemagglutinin, fimbriae, outer membrane proteins, adenylate cyclase, and hemolysin which appear to be involved in the pathogenesis of whooping cough (97, 149). Two related forms of coordinate regulation of virulence factor expression are observed in this organism, antigenic or phenotypic modulation and phase variation. A complete lack of expression of the majority of B. pertussis virulence factors is noted in the modulated state or avirulent phase. Antigenic modulation occurs when the bacterium is exposed to certain environmental conditions such as increased concentration of magnesium sulfate and nicotinic acid or decreased growth temperature (82). This phenotypic change is rapidly reversible when normal growth requirements are restored. In a separate event, B. pertussis can undergo a reversible, metastable genetic change to an avirulent phase (134, 148). The frequency of the shift from virulent to avirulent phases differs between B. pertussis strains but is generally from 10^{-3} to 10^{-6} .

The bvg (for Bordetella virulence gene) or vir (for virulence) locus is required for coordinate regulation of expression of the

majority of virulence factors in B. pertussis (4, 118, 133, 134). The locus contains two genes (bvgA and bvgS) which encode proteins homologous to a family of sensor-receiver, two-component regulatory systems found in other bacterial species (4, 81, 93, 121, 135). Investigators have recently proposed a model for the function of the regulatory system in which the BvgS protein acts by sensing or receiving environmental stimuli and transmitting information to the BvgA protein which is a positive regulator of transcription of the virulence-associated genes (4, 121). The BvgS protein appears to function as a protein kinase and activates BvgA by phosphorylation. According to the model, modulating signals would reversibly inhibit activation of virulence-associated gene transcription while removal of the environmental stimuli would allow expression of the virulence factors. The change to the avirulent phase is due to a rare frameshift mutation in the bvg locus that removes the trans-activating signal (134). Reversion to the virulent phase is due to back mutation which restores the correct reading frame of the gene. It appears, however, that the regulatory system is more complex than this simplified model suggests and clarification awaits further research (79, 84, 118, 121).

Antigenic modulation and phase variation are observed in B. bronchiseptica and B. parapertussis (40, 68, 85, 109) and both species have bvg loci that are nearly identical to the B. pertussis locus (58, 95). Though examples of phase variation and antigenic modulation exist in B. avium (47, 49), the relationship of the coordinate regulatory system of B. avium to the bvg system in the other members of the genus

Bordetella has not been determined. The virulence factors of B. avium that have been characterized include adhesins, toxins, and outer membrane proteins.

Adhesins

Adhesins are bacterial surface structures or molecules which mediate adhesion of bacteria to target cell surfaces by interacting specifically with receptors present on the eucaryotic cell membrane or glycocalyx (8). Bacterial adhesion is an essential early step in infection and is especially important for mucosal pathogens which must avoid being swept away by the mucosal surface protective mechanisms of the host (104). By maintaining bacteria on the mucosal surface in intimate contact with the epithelium, the adhesins also help to direct local cellular toxicity and function in invasion of host cells by intracellular pathogens. Because adhesion is a specific interaction, the presence of a bacterial adhesin and the corresponding receptor in a tissue or host is thought to be at least partially responsible for tissue tropism and host specificity of many bacterial pathogens (138).

Like other members of the genus Bordetella, B. avium exhibits a tropism for the ciliated respiratory epithelium (8, 9, 10, 92, 138, 140). Two putative virulence factors which may contribute to adhesion of B. avium to the epithelium are fimbriae and a hemagglutinin. Fimbriae (pili) projecting from the surface of B. avium have a diameter of 2.0 nm and range in length from 370 nm to 1500 nm (69). These filamentous structures are composed of a polymer of a single protein

subunit with a reported molecular weight of 13,100. Purified fimbriae isolated from virulent B. avium adhered to the epithelium of turkey tracheal organ cultures and hyperimmune antiserum against the fimbriae blocked in vitro adherence of B. avium to the tracheal explants. Poults immunized with the purified fimbriae were protected from clinical signs of turkey bordetellosis and showed decreased colonization of the sinuses and trachea by B. avium (2).

Mooi et al. (98) using Western immunoblot analysis showed that antiserum raised against the serotype 2 fimbrial subunit of B. pertussis cross-reacted with three B. avium polypeptides with molecular weights of 14,400, 17,000, and 24,000 (14.4K, 17K, and 24K polypeptides), indicating the possible presence of different fimbrial subunits in B. avium. In contrast, an oligonucleotide probe derived from the serotype 2 fimbrial gene did not hybridize to any B. avium DNA, suggesting that though there is antigenic relatedness, the gene for the serotype 2 fimbriae is divergent from the B. avium fimbrial gene. Antiserum prepared against the other major fimbrial type of B. pertussis, the serotype 3 fimbriae, did not cross-react with any B. avium proteins.

Adhesins responsible for agglutination of erythrocytes are termed hemagglutinins. If the receptor on the surface of the target cell resembles the hemagglutinin receptor on the erythrocyte, the hemagglutinin may be involved in bacterial attachment to target cells during infection. Virulent isolates of B. avium have the ability to agglutinate guinea pig erythrocytes (14, 73). The hemagglutinin appears to be a nonfimbrial protein but further molecular characterization has

not been done (69, 128). Hemagglutination-negative mutants exhibited reduced adherence to turkey tracheal mucosa using an in vivo model while reversion of one mutant to hemagglutination-positive status resulted in the return of much of the ability to adhere (14). Though two hemagglutinins, the filamentous hemagglutinin and pertussis toxin, are primary adhesins of B. pertussis (138, 139), no evidence of either has been found in B. avium (3, 49). Recently, reduced-adherence B. avium mutants were shown to be lacking a 46K protein produced by the wild-type strain (25, 26). The relationship of this protein to either the fimbriae or the hemagglutinin of B. avium has not been determined.

Arp et al. (13) have indicated that the turkey tracheal mucosal receptor for the B. avium adhesin closely resembled GD_{1a} and GT_{1b} gangliosides. The most prominent component of the receptor appeared to be N-acetylneuramic acid. Glycoconjugates containing N-acetylneuramic acid also acted as receptors on swine nasal mucosa for B. bronchiseptica (67) were not important as human cilia receptors for B. pertussis (141).

Toxins

The tracheal lesions observed in turkeys are consistent with the involvement of bacterial toxins in the pathogenesis of turkey bordetellosis (10). In addition, in vitro studies of B. avium have demonstrated cytotoxicity for the ciliated tracheal epithelium. When incubated with bacterial suspensions, infected turkey tracheal organ cultures showed rapid changes including ciliostasis, hydropic degeneration and sloughing of the epithelial cells (55). This cytotoxic

activity correlated with in vivo bacterial virulence (54, 55, 92) and ability of the bacterial isolate to adhere to tracheal organ culture (91). In another study using an in vivo model, cytopathologic changes occurred more slowly and closely mimicked the changes seen in turkeys with bordetellosis (10). The factor(s) responsible for cytotoxicity has not been specifically identified but several toxins of B. avium have been described which could potentially contribute to pathologic changes caused by the organism.

The tracheal cytotoxin, first described in B. pertussis, is present in all Bordetella spp. and in B. avium-like organisms (49, 51). The toxin is an anhydropeptidoglycan monomer with a molecular mass of 921 daltons that is released extracellularly by growing cells (34, 35, 50). The cytotoxin has been shown to cause ciliostasis and extrusion of ciliated cells in hamster tracheal organ cultures, mimicking the bacterial-induced changes seen in vivo (35, 50). The toxin also inhibited DNA synthesis in hamster tracheal epithelial cell culture. The inhibition might slow replacement of extruded ciliated cells in the trachea and be responsible for the extended clinical course observed in diseases caused by members of the genus Bordetella. Though tracheal cytotoxin has recently been purified to homogeneity (35), the toxin's effects on turkey tracheal epithelium have not been examined.

Virulent B. avium isolates produce a cell-associated, 155K protein with biological activity similar to dermonecrotic or heat-labile toxin of other members of the genus Bordetella (49, 114). This toxin causes necrotic skin lesions in guinea pigs and turkeys injected intradermally

and is lethal for mice, guinea pigs, and turkey poults (49, 113, 114, 115). However, incubation of the toxin with turkey tracheal-ring organ cultures caused neither ciliostasis (115) nor reduction in tracheal epithelial cell metabolism (142). The role of B. avium dermonecrotic toxin in pathogenesis of turkey bordetellosis is unknown. Gentry-Weeks and Curtiss have cloned the dermonecrotic toxin gene of B. avium and expressed the recombinant protein in Escherichia coli (46). This should allow direct comparison of the B. avium toxin with the dermonecrotic toxins of other Bordetella spp.

Two other toxins or factors have been described in B. avium, a heat-stable toxin and a histamine-sensitizing factor. The heat-stable toxin is loosely cell-associated or excreted into the medium (126). Crude preparations of heat-stable toxin are lethal to mice but cause only decreased thymus weights in turkey poults (126, 152). It appears that much of the toxin's biological activity is due to lipopolysaccharide (83). A study by Simmons et al. (129) indicated virulent B. avium isolates produced a factor with histamine-sensitizing properties similar to the pertussis toxin of B. pertussis (147). Because pertussis toxin is not present in B. avium (3, 49) this histamine-sensitizing activity must be due to some other undefined factor present in the turkey pathogen.

Outer membrane proteins

The cell wall of gram-negative bacteria consists of three layers: the inner or cytoplasmic membrane, the mechanically rigid peptidoglycan

layer, and a highly specialized outer membrane which is composed of protein, phospholipid, and lipopolysaccharide and which is closely associated with the peptidoglycan layer (90, 101). Proteins make up nearly half of the total mass of the outer membrane and function as enzymes, receptors for bacteriophage, and adhesins (18, 90, 101, 105). Certain outer membrane proteins (OMPs) play an important role in membrane permeability allowing the passage of nutrients from the medium and preventing entry of toxic substances such as antibiotics into the cell. Other proteins function by stabilizing the outer membrane and attaching the membrane to the peptidoglycan. Because the bacterial surface comes in direct contact with host cells, proteins present in the outer membrane might play an important role in colonization and subsequent immunity.

Characterization of OMPs requires experimental procedures for separation of proteins of interest from contaminating bacterial compounds. Numerous methods for isolation of OMPs have been described and differ according to the bacterial species and the individual proteins being studied (90). Many isolation procedures take advantage of biochemical differences that exist between inner and outer membranes. A difference in membrane densities allows separation of the inner and outer membranes in cell envelope preparations by sucrose gradient ultracentrifugation (122). Treatment of isolated bacterial envelopes with the detergents, Triton X-100 (123) and sodium lauryl sarcosinate (Sarkosyl)(43) selectively solubilizes the cytoplasmic membrane while leaving the outer membrane and peptidoglycan relatively intact. Both

detergents have been successfully used for isolation and characterization of OMPs from many bacteria including B. pertussis (106, 111, 117) and B. avium (8, 60, 61, 88). Outer membrane proteins have been extracted from the surfaces of intact bacteria with procedures using the detergent Zwittergent-3,14 (23, 99), lithium acetate (74), acid glycine (102), and sodium chloride-sodium citrate (136). Outer membrane vesicles or blebs containing proteins and lipopolysaccharide are shed into the culture medium by certain gram-negative bacteria and can easily be separated from the whole cells for biochemical analysis (36, 107, 151). A limitation of this technique for examination of OMPs is that the vesicles frequently differ slightly in composition from the outer membrane of intact cells (107, 151).

Three classes of major OMPs present in many gram-negative bacteria are lipoproteins, OmpA-like proteins, and porins. Lipoproteins have been described in several gram-negative species (31, 39, 52, 94) but the most extensively characterized lipoprotein is the murein lipoprotein of E. coli (28). This protein is a small, 7.2K molecule that exists in a large number of copies in the outer membrane (28, 101). Murein lipoprotein is covalently bound to the peptidoglycan layer of E. coli and is thought to function by stabilizing the outer membrane.

Another class of major proteins found in the outer membrane of many gram-negative bacteria contains peptides that are phenotypically or genetically related to the OmpA protein of E. coli (90, 105). The OmpA protein is a transmembrane protein that is closely associated with the peptidoglycan layer and is heat-modifiable. The protein has a

nondenatured apparent molecular weight of 28,000 and a heat-modified molecular weight of 35,000. The OmpA protein, along with murein lipoprotein, appears to function in maintaining the structural integrity of the cell wall. Similar OMPs have been identified in members of the family Enterobacteriaceae and a variety of other gram-negative bacteria (17, 24, 146).

Porins are proteins that have molecular masses of from 30,000 to 50,000 daltons and are generally organized in the outer membrane as trimers of three identical subunits (18). The proteins may be constitutively present on the outer membrane or they may be expressed only under certain environmental conditions. Porins have been separated into two classes based on solute permeability. General diffusion porins form relatively nonspecific diffusion channels which sort solutes mainly based on size, usually excluding molecules with molecular masses of greater than approximately 650 daltons. All gram-negative bacteria examined have at least one type of general diffusion porin present in the outer membrane and some have a second type which is specific for only one or a few classes of solutes (18). Binding sites present in the channel of specific porins regulate permeability for a limited number of solutes. Examples of specific-purpose porins include LamB of E. coli (101) and protein P of Pseudomonas aeruginosa (18, 19) which selectively allow passage of maltose and phosphate, respectively. The 40K major outer membrane protein of B. pertussis is present in the membrane as a trimer and functions as an anion-selective porin (7, 78). The porin protein appears to be constitutively expressed in all isolates,

including phenotypically-modulated and avirulent-phase organisms. Similar molecular-weight major proteins have been present in the OMP profiles of each B. bronchiseptica, B. parapertussis, and B. avium isolate examined (6, 40, 88).

The outer membrane contains, in addition to a few classes of major proteins, numerous minor protein species, some of which are present in crude OMP preparations in amounts below the level of routine detection methods (18, 90). The minor OMPs can have important functions such as iron acquisition (57) and vitamin uptake (75). Under certain growth conditions some minor OMPs are induced and become abundant in the membrane (30, 45).

The OMPs of B. pertussis, B. parapertussis, and B. bronchiseptica have been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)(40, 108, 117) and by surface labeling techniques (6, 106, 107, 109, 111). The three species have typical gram-negative outer membranes with a small number of major proteins and numerous minor proteins. The OMPs from virulent isolates show little variation within species and between serotypes (6, 40, 108, 117). Though certain proteins are shared by B. pertussis, B. bronchiseptica, and B. parapertussis, overall patterns are distinct between species. The OMP profiles of all three species contain proteins that appear to be coordinately-controlled and are present only in the virulent, nonmodulated bacteria (40, 109). The effects of phenotypic modulation and phase variation on OMP production have been examined extensively in B. pertussis and at least four proteins have been shown to be lacking in

the outer membrane of avirulent-phase and antigenically-modulated cells (6, 40, 108, 117). Except for one protein, the 69K protein, the role of the virulence-associated OMPs of B. pertussis in pathogenicity has not been examined.

A bvg-regulated 69K OMP of B. pertussis has been shown to be immunoprotective for mice (32, 124). Both B cell (53, 137) and T cell (38) responsiveness to the antigen have been demonstrated in humans with previous pertussis infection. The protein is found in all virulent B. pertussis isolates and is nonfimbrial in nature (29). The 69K OMP is immunologically cross-reactive with B. bronchiseptica and B. paraptussis antigens with molecular masses of 68,000 and 70,000 daltons, respectively (96). Antibody to the 68K protein passively protected mice from virulent B. bronchiseptica challenge (96) and in piglets from vaccinated sows the presence of antibody to the 68K OMP correlated with the degree of protection from atrophic rhinitis and pneumonia (80, 103). It has not been determined if B. avium has a similar OMP; however, Weiss et al. using monoclonal antibodies to the 68K and 69K OMPs found no cross-reacting antigens in one virulent B. avium isolate (150).

Characterization of the 69K OMP has indicated it may function as an adhesin for B. pertussis (86, 87). Cloning and sequencing of the gene for the protein has revealed two arginine-glycine-aspartic acid (RGD) tripeptides (32). The RGD sequence is found in fibronectin and other eucaryotic extracellular matrix proteins and functions as a recognition site for the cell-surface integrin receptor superfamily (66). The

recognition sequences in the 69K protein appear to be important in bacterial attachment and invasion of tissue culture cells (86). Mimicry of host proteins to take advantage of preexisting receptors on the target cells is a common theme for pathogens. Several pathogens contain proteins with RGD recognition sequences that are involved in cellular attachment (44), most notably, a loosely associated surface protein of B. pertussis, the filamentous hemagglutinin contains an RGD sequence which is involved in bacterial attachment to the integrin CR3 receptor of human macrophages (112).

Initial examination of total cellular proteins (65), soluble proteins (77), and total cell-wall proteins (73) of virulent B. avium isolates by SDS-PAGE indicated no differences in protein profiles among strains belonging to different serotypes or isolated from diverse geographical areas. Similarly, examination of outer membrane-enriched fractions from numerous virulent isolates showed no major differences in electrophoretic mobility of the OMPs (61, 88). Homogeneity of OMP profiles within a species appears to be characteristic of all Bordetella spp., but is not seen in all bacteria. In certain species of gram-negative bacteria, the OMP profiles can differ according to serotypes and biotypes (1, 15, 110).

Outer membrane protein profiles from virulent B. avium isolates contained major 21K and 37K bands and at least 10 bands which stained less-intensely with molecular weights ranging from less than 14,000 to 139,000 (88). Hellwig et al. compared profiles from virulent, adherent isolates with a reduced-adherence mutant and showed decreased amounts of

several OMPs in the mutant (61). Growth at a reduced temperature appeared to decrease or eliminate expression of many of the OMPs of B. avium. Spontaneous phase variants of a virulent B. avium strain have been isolated and found to lack the 20K, 38K, 50K, and 93K OMPs (47). The proteins that were not expressed in the phase variants corresponded to those lacking in cells phenotypically modulated by growth in the presence of nicotinic acid or magnesium sulfate. These results indicate control of expression of certain OMPs of B. avium might be similar to the bvg system found in the other members of the genus Bordetella.

The local and systemic immune response to the OMPs of B. avium has been examined by Western immunoblot analysis using sera and tracheal washings from experimentally infected turkeys. Bordetella avium OMPs with molecular weights of from 18,000 to 25,000 were recognized by antibodies in sera from 3-week-old experimentally infected turkeys (11). In addition, both convalescent sera and tracheal washings recognized 50K and 110K OMPs. In another study using a different virulent B. avium isolate, reactivity of antibodies in sera with OMPs was observed at two weeks post infection and increased during the following two weeks (60). At four weeks post inoculation, eight OMPs ranging from less than 14K to 100K were recognized by sera with much of the reactivity directed to the major 21K OMP. Immunoreactivity of tracheal washings was similar but less intense when compared with the sera.

Information available on the OMPs of B. avium indicates their potential importance in pathogenesis of turkey bordetellosis. The work described herein was intended to further characterize the proteins in

the outer membrane of B. avium. The OMP profiles of numerous B. avium isolates and representative strains from other members of the genus Bordetella were compared by SDS-PAGE. The OMPs of B. avium were evaluated for heat-modifiability, association with the peptidoglycan layer, cell-surface exposure, and expression under different growth conditions. Finally, the amino acid composition and the amino-terminal amino acid sequence of the 21K OMP of B. avium was determined.

SECTION I. OUTER MEMBRANE PROTEINS OF BORDETELLA AVIUM

SUMMARY

The outer membrane proteins of Bordetella avium were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sarkosyl-insoluble outer membrane protein-enriched profiles from 50 virulent B. avium isolates were very similar, containing major 21,000- and 37,000-molecular-weight proteins (21K and 37K proteins) and at least 13 less-intensely stained proteins with molecular weights ranging from 13,500 to 143,000. Heat-modifiability of the outer membrane proteins of B. avium was examined by varying the solubilization temperature prior to electrophoresis. Certain proteins, including the major 21K and 37K proteins, were heat-modifiable and showed increased mobility at higher solubilization temperatures. The 21K, 27K, 31K, and 37K outer membrane proteins were noncovalently associated with the underlying peptidoglycan layer. It was necessary to treat cell envelopes with 2% sodium dodecyl sulfate and temperatures in excess of 60°C for 15 min to release the murein-associated proteins. Exposure of proteins on the cell surface of B. avium was assessed by labeling with ¹²⁵I followed by electrophoresis. As many as 13 bands were present in profiles from labeled whole cells. Of the surface-labeled bands, eight corresponded to bands in a radiolabeled outer membrane preparation. The outer membrane protein profile of B. avium was compared to profiles from other Bordetella spp., including 20 B. avium-like organisms and 16 B. bronchiseptica strains isolated from turkeys. The outer membrane protein profile of B. avium was distinctly different from that of the other bordetellae. The effect

of variations in the growth medium on the expression of outer membrane proteins of B. avium was examined. Expression of 22K, 24K, and 56K proteins was decreased by addition of either 20 mM MgSO_4 or 500 ug of nicotinic acid per ml to media, conditions known to inhibit expression of certain outer membrane proteins in B. pertussis.

INTRODUCTION

Bordetella avium is the etiologic agent of turkey bordetellosis, a highly contagious upper respiratory disease of poults characterized by oculonasal discharge, sneezing, dyspnea, decreased weight gain, and tracheal collapse (5, 17, 41, 44). Uncomplicated bordetellosis in turkeys generally results in high morbidity and low mortality but disease under field conditions is often more severe with increased mortality possibly due to infection by secondary pathogens (41) and increased stress (45). Like other members of the genus Bordetella, B. avium exhibits a tropism for ciliated epithelium of the upper respiratory tract (5, 6). Colonization of the trachea results in inflammation with loss of epithelium and distortion of the mucosa and tracheal rings. Isolation of two other related bacteria is frequent from turkeys with upper respiratory disease. One group of bacteria identified as B. bronchiseptica appears nonpathogenic in experimentally infected birds (26). The other organism is phenotypically similar to B. avium but is avirulent and has been temporarily designated B. avium-like by Jackwood et al. (25).

Little is known about the bacterial surface structures of B. avium and their involvement in pathogenesis of turkey bordetellosis. Surface appendages include peritrichous flagella and fimbriae (24, 27). Bordetella avium possesses a poorly characterized hemagglutinin which, along with fimbriae, might play a role in adhesion to ciliated respiratory epithelial cells (8, 24). Outer membrane proteins (OMPs)

from a limited number of B. avium isolates have been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22). No apparent differences were observed in OMP profiles from virulent B. avium strains but a hemagglutination-negative isolate lacked certain high-molecular-weight OMPs. Sera and tracheal washings from experimentally infected turkeys recognized at least eight OMPs as determined by Western immunoblot analysis (7, 21). Reactivity to a 21,000-molecular-weight OMP (21K OMP) was especially intense with antibodies to this protein found in both sera and tracheal washings.

Initial work has indicated the potential importance of B. avium OMPs in pathogenesis of turkey bordetellosis. In this study, OMP profiles from numerous B. avium isolates were obtained by detergent extraction of cell envelopes and compared with profiles from other Bordetella spp. including B. bronchiseptica and B. avium-like organisms isolated from turkeys. The OMPs of B. avium were evaluated for heat-modifiability, cell-surface accessibility to radioiodination, and noncovalent association with the underlying peptidoglycan layer. Finally, the effect of growth conditions on OMP expression by B. avium was determined by varying incubation time and growth media.

(A preliminary report of these findings was presented at the 70th Annual Meeting of the Conference of Research Workers in Animal Disease, Chicago, 1989, abstr. no. 69.)

MATERIALS AND METHODS

Bacterial Strains

Bacteria used for this study are listed in Table 1. In addition to the strains listed in Table 1, OMPs were examined from 41 B. avium, 18 B. avium-like, and 16 B. bronchiseptica strains isolated from turkeys with upper respiratory disease in Iowa, California, North Carolina, Ohio, Minnesota, and Wisconsin. Stock cultures of all strains were maintained at -80°C during the course of this study.

Media and Growth Conditions

Bordetella avium isolates were grown on brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) agar (BHIA) containing 1.5% agar (BiTek agar; Difco) for 24 h at 37°C and passed to BHIA or BHI broth (BHIB). To evaluate the effect of incubation time on protein expression B. avium strain 75 was incubated at 37°C for 24, 36, or 48 h on BHIA or in BHIB prior to isolation of OMPs. To further evaluate the effect of growth medium on expression of OMPs by B. avium, strain 75 was also grown in modified Stainer-Scholte broth (SSM)(42) as adapted for growth of B. avium by Gentry-Weeks et al. (16). Bacteria were transferred from BHIA and passaged twice in SSM or in SSM containing 500 ug of nicotinic acid (Sigma Chemical Co., St. Louis, Mo.) per ml or lacking NaCl and supplemented with 20 mM MgSO₄ (Fisher Scientific, Fair Lawn, N. J.) at 37°C for 24 h each and used for OMP isolation. All broth cultures

Table 1. Bacterial strains

Strain	Virulence ^a	Origin (reference)
<u>Bordetella avium</u>		
75	+	L. H. Arp, Iowa (5)
ATCC 35086	+	Type strain (27)
838	+	L. H. Arp, Iowa (10)
ATCC 31770	+/- ^b	Vaccine strain, American Scientific Laboratories, Wis. (13)
NCD	+	D. G. Simmons, N. C. (43)
NCD-1	-	Avirulent strain derived from NCD, D. G. Simmons, N. C. (30)
W	+	D. G. Simmons, N. C. (43)
197	+	Y. M. Saif, Ohio (26)
F9000336	NT ^c	R. P. Chin, Calif.
<u>Bordetella avium-like</u>		
023	-	Y. M. Saif, Ohio (26)
101	-	Y. M. Saif, Ohio (26)
<u>Bordetella pertussis</u>		
536	NT	Virulent, E. Tuomanen, N. Y. (37)
537	NT	Avirulent-phase variant of 536, E. Tuomanen, N. Y. (37)
<u>Bordetella bronchiseptica</u>		
469	NT	Host species: human, E. Tuomanen, N. Y.
Bailey	NT	Host species: swine, R. Ross, Iowa
103	-	Host species: turkey, Y. M. Saif, Ohio (26)
<u>Bordetella parapertussis</u>		
504	NT	Virulent, E. Tuomanen, N. Y.

^aVirulence for turkey poults.

^bMildly virulent for turkeys (26).

^cVirulence for turkey poults has not been tested.

were incubated in a shaking incubator at 210 rpm (model G25-KC; New Brunswick Scientific Co., Inc., Edison, N. J.).

Strains used for comparison of OMP profiles among different Bordetella spp. were passaged twice on Bordet-Gengou agar (Difco) containing 15% defibrinated sheep blood and 1% glycerol (BGA) at 37°C. Bordetella pertussis strains were grown for 72 h, B. parapertussis and B. bronchiseptica isolates were grown for 48 h, and B. avium and B. avium-like strains were grown for 36 h on BGA.

All bacteria were harvested from plates in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; pH 7.4) buffer (Sigma) or from broth and collected by centrifugation at 10,000 x g. The bacteria were washed once with 10 mM HEPES (pH 7.4) buffer and approximately 1.5 gm (wet weight) of cells were resuspended in 15 ml of buffer and stored at -80°C. All cells were used within two weeks for isolation of OMPs or peptidoglycan-associated proteins.

OMP Preparation

The OMP-enriched fractions were prepared by a modification of the procedure of Hellwig and Arp (21). Briefly, the cells were thawed and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to a final concentration of 0.1 mM. The cells were disrupted by a sonicator (model 350; Branson Sonic Power Co., Danbury, Conn.) for 10, one minute bursts (50% cycle, power setting 7) while cooling in an ice water bath. Whole cells and large debris were removed by centrifugation

at 5,000 x g for 20 min and the total membrane fraction was harvested from the supernatant by centrifugation at 100,000 x g for 60 min at 4°C. The membrane fraction was suspended in 1% (wt/vol) Sarkosyl (sodium lauryl sarcosine; International Biotechnologies, Inc., New Haven, Conn.) in 10 mM HEPES (pH 7.4) buffer containing 0.1 mM PMSF for 30 min at room temperature. The detergent-insoluble material containing the OMP-enriched fraction was harvested by centrifugation at 100,000 x g for 60 min at 4°C. The final insoluble pellet was suspended in deionized water to a concentration of 1.0 to 8.0 mg of protein per ml and stored at -80°C. Protein concentrations were determined by the bicinchoninic acid assay (36; Pierce Chemical Company, Rockford, Ill.) using bovine serum albumin as a standard.

Radioiodination

Whole cells and OMPs from B. avium 75 were radiolabeled with ¹²⁵I essentially as described by Richardson and Parker (38). Bacteria to be radiolabeled were grown on BHIA or in BHIB for 36 h at 37°C. Whole cells for surface radioiodination were washed twice with phosphate-buffered saline (PBS; 0.0132 M phosphate, 0.15 M NaCl; pH 7.3) and resuspended to 0.1 gm (wet weight) per ml of PBS. The OMPs were isolated from bacteria grown on BHIA or in BHIB as described above and diluted to 50 ug of protein concentration per ml of PBS. Iodo-beads (four beads per whole cell reaction and two beads per OMP reaction) were used as the catalyst for radioiodination. The Iodo-beads were rinsed

according to the procedure from the supplier (Pierce) and added to polypropylene tubes containing 1 mCi of Na^{125}I (Amersham Corp., Arlington Heights, Ill.). After the beads were incubated at room temperature for 5 min, 1 ml of whole cell or OMP suspensions was added to each tube and radioiodination was allowed to proceed for 5 min. The reaction was stopped by removal of the whole cell and OMP suspensions from the Iodo-beads and unreacted ^{125}I was removed from whole cells by centrifugation (38) and from OMP preparations using a desalting column (Econopac-Pac 10DG; Bio Rad Laboratories, Richmond, Calif.).

SDS-PAGE

A modified Laemmli (29) procedure was used to separate proteins on discontinuous gels consisting of 4% stacking gels and 6 to 20% gradient resolving gels. Electrophoresis was carried out on a vertical slab apparatus (Bio-Rad) following the procedure in the suppliers catalog. Proteins were solubilized in sample buffer containing 2% SDS, 5% 2-mercaptoethanol (2-ME), 20% glycerol, 0.001% bromophenol blue, and 0.0625 M Tris (pH 6.8) at 100°C for 5 min unless otherwise stated. The effect of the reducing agent was examined by comparing migration of proteins solubilized in the above sample buffer with those solubilized in an identical buffer without 2-ME. Each lane was loaded with either 5 to 20 ug of protein or ca. 40,000 cpm. After electrophoresis, gels were either stained with Coomassie brilliant blue R250 for visualization of protein bands or were fixed with 40% methanol-10% acetic acid, dried

onto Whatman 3MM paper, and exposed to X-ray film (X-OMAT AR; Eastman Kodak Co., Rochester, N. Y.). The following proteins were used as molecular size standards (Life Technologies, Inc., Gaithersburg, Md.): lysozyme (14,300), B-lactoglobulin (18,400), carbonic anhydrase (29,000), ovalbumin (43,000), bovine serum albumin (68,000), phosphorylase B (97,400), and myosin (H-chain)(200,000). Apparent molecular weights were determined by comparison with the protein standards according to standard procedures (19).

Peptidoglycan-associated proteins were isolated as described by Armstrong and Parker (2). A total cell membrane pellet obtained from 2.5 gm (wet weight) of whole cells was suspended in 15 mM Tris (pH 8.0) buffer containing 2% SDS and equal fractions were heated at 37°C, 50°C, 60°C, 80°C, or 100°C for 15 min. The insoluble material consisting of crude peptidoglycan and associated complexes was pelleted by centrifugation at 100,000 x g for 1 h at 20°C. Each pellet was washed twice in 10 mM HEPES (pH 7.4) buffer containing 0.1 mM PMSF and was suspended in 0.5 ml of deionized water. The samples (10 ul per lane) were treated by heating in SDS-PAGE sample buffer at 100°C for 5 min and the solubilized proteins were separated by SDS-PAGE as described previously.

RESULTS

SDS-PAGE of Sarkosyl-insoluble Proteins of B. avium

Sarkosyl-insoluble OMP fractions from 50 B. avium isolates grown on BHIA for 36 h were compared by SDS-PAGE. Two bands representing the 21K and 37K OMPs were the most prominent bands in all isolates. At least 11 less-intensely stained bands (13.5K, 15K, 18K, 23K, 27K, 31K, 54K, 73K, 80K, 83K, and 143K) were apparent in the OMP profile from strain 75 (Figure 1, lane B). No differences in the relative concentration or mobility of the major 21K and 37K bands were observed between strains and little variation was noted in the less-intensely stained bands (Figure 1). Two less-intensely stained bands, the 41K and 43K bands, were not apparent in the strain 75 profile but were observed in certain isolates. Similar OMP profiles were observed in strain NCD and its avirulent derivative NCD-1 (Figure 1, lanes F and G). In addition to the strains shown in Figure 1, 23 other B. avium isolates were examined and all had identical or nearly identical OMP profiles (data not shown).

Because strains initially used for isolation of OMPs in our laboratory had been obtained from infected birds at least six years previously, we included 19 isolates from field outbreaks of upper respiratory disease in turkeys during 1989 and 1990 to determine if B. avium OMP profiles had changed from earlier isolates. No differences in the OMP profiles were noted between the two groups of isolates. The

Figure 1. SDS-PAGE analysis of OMPs from the following B. avium isolates grown on BHIA for 36 h at 37°C: lane B, isolate 75; lane C, isolate 838; lane D, type strain ATCC 35086; lane E, vaccine strain ATCC 31770; lane F, isolate NCD; lane G, isolate NCD-1, an avirulent derivative of NCD; lane H, isolate W; lane I, isolate 197; lane J, isolate F9000336. Lane A, molecular size standards as described in the text.

A B C D E F G H I J

200-

97.4-

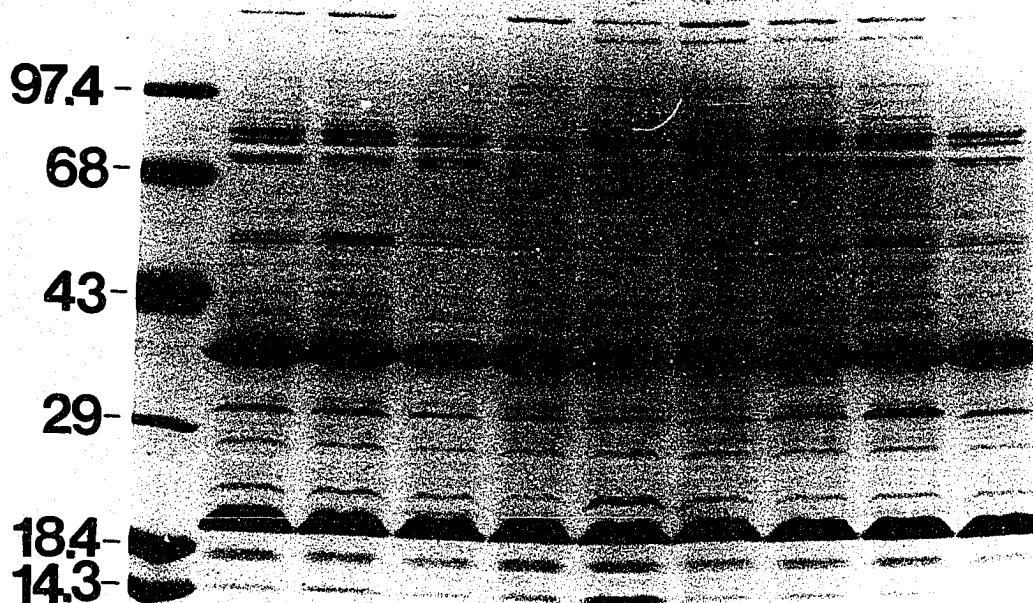
68-

43-

29-

18.4-

14.3-

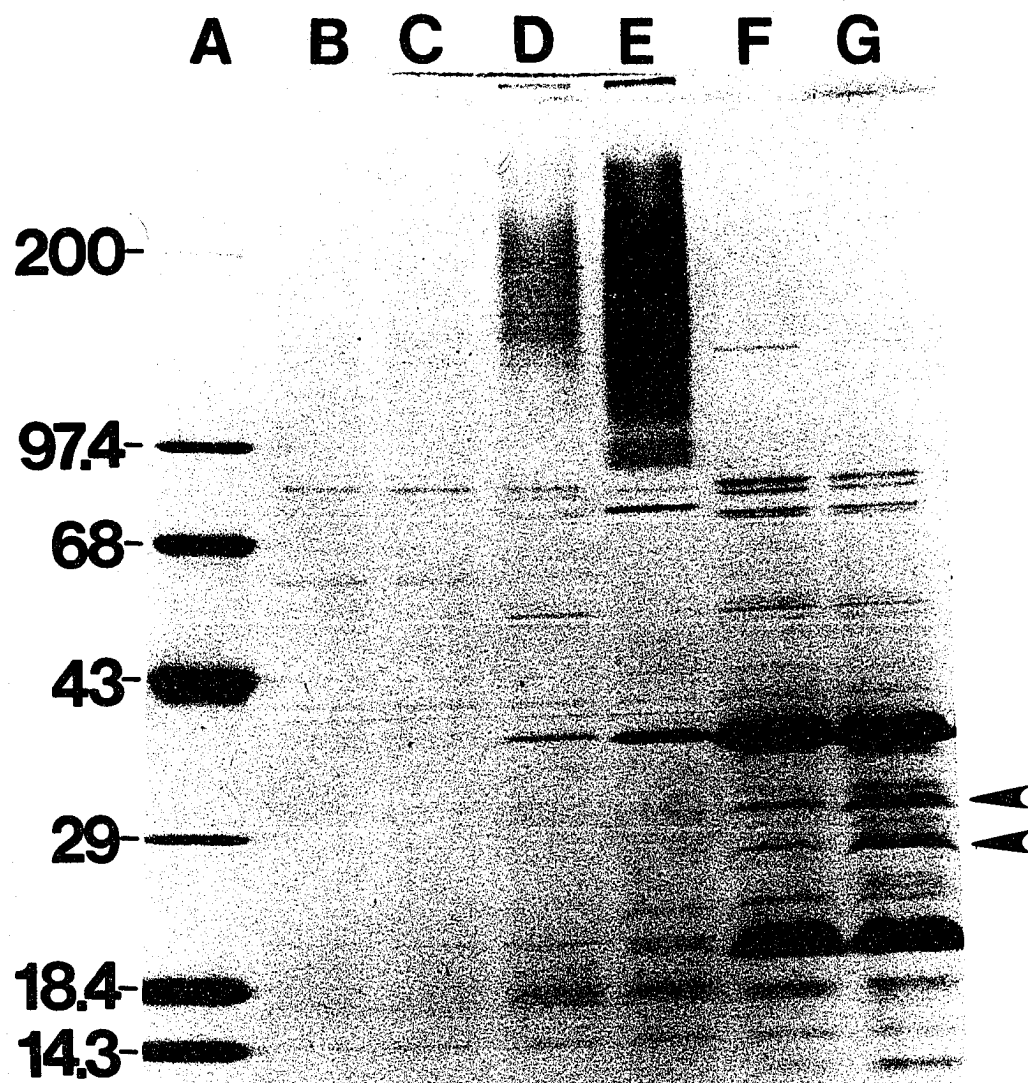


profile from strain F9000336 is representative of OMPs obtained from recent field isolates (Figure 1, lane J).

Heat-modifiable OMPs of B. avium

Heat-modifiability of B. avium strain 75 Sarkosyl-insoluble proteins was examined by varying the temperature and time of solubilization in SDS-PAGE sample buffer containing 2-ME prior to electrophoresis. The majority of the OMPs were not solubilized in sample buffer incubated at room temperature and 37°C as indicated by the relatively low amount of protein observed in the resolving gel in lanes B and C of Figure 2. Increased protein staining was noted in the high-molecular-weight region of the gel at solubilization temperatures of 60°C and 80°C (Figure 2, lanes D and E). The major 21K and 37K bands were first visible in the gel when samples were solubilized at 80°C and 60°C, respectively. The major bands increased in intensity with temperatures up to 100°C but incubation for 30 min at 100°C in sample buffer prior to electrophoresis had no apparent effect on the intensity of either band. The minor 27K and 31K bands were not apparent below the 100°C solubilization temperature and increased in intensity when the solubilization time was lengthened to 30 min (indicated by arrows in Figure 2). A 75K band was observed at a solubilization temperature of 80°C but was not present at other temperatures. Omission of the reducing agent 2-ME from the SDS-PAGE sample buffer appeared to have no effect on OMP mobility (data not shown).

Figure 2. SDS-PAGE of OMP-enriched fractions from B. avium 75 solubilized at different temperatures and for different times in sample buffer (described in text) prior to electrophoresis. Solubilization conditions: lane B, room temperature for 5 min; lane C, 37°C for 5 min; lane D, 60°C for 5 min; lane E, 80°C for 5 min; lane F, 100°C for 5 min; lane G, 100°C for 30 min. Ten micrograms of protein was applied to each lane. Arrows indicate the heat-modifiable 27K and 31K OMPs. Lane A, molecular size standards as described in the text.



Peptidoglycan-associated Proteins

Noncovalent association of certain OMPs with the underlying peptidoglycan layer can affect heat-modifiability of these proteins. Total cell envelope fractions from B. avium 75 grown on BHIA for 36 h were incubated in 2% SDS at 37°C, 50°C, 60°C, 80°C or 100°C for 15 min to release proteins that were noncovalently associated with the crude peptidoglycan. The 21K, 27K, and 31K proteins were associated with the peptidoglycan at lower temperatures but were totally dissociated from the crude murein layer when treated in SDS at temperatures in excess of 60°C (Figure 3, lane F). The majority of the 37K murein-associated protein, corresponding to the major 37K OMP, was released from the peptidoglycan by incubation at 80°C and was totally solubilized by treatment in SDS at 100°C prior to SDS-PAGE (Figure 3, lanes F and G). Other OMPs were not as closely associated with the peptidoglycan and were released from the crude peptidoglycan fraction by incubation at room temperature.

SDS-PAGE of Radiolabeled Proteins

Examination of radioiodinated OMPs from B. avium strain 75 by SDS-PAGE routinely showed nine bands that corresponded to the 13.5K, 15K, 18K, 21K, 27K, 31K, 37K, 54K, and 80K OMP bands seen in Coomassie blue-stained gel (Figure 4, lanes B and D). The only consistent difference between radiolabeled OMPs from cells grown on BHIA and BHIB was the increased intensity of the lower-molecular-weight bands from cells grown

Figure 3. SDS-PAGE analysis of peptidoglycan-associated proteins. Cell envelope preparations from B. avium 75 were treated at different temperatures in 2% SDS for 15 min. The insoluble material containing peptidoglycan and associated protein was incubated in SDS-PAGE sample buffer at 100°C for 5 min before electrophoresis. Lane B, cell envelope fraction with no SDS treatment before SDS-PAGE. Cell envelope fractions treated with SDS at 37°C, 50°C, 60°C, 80°C, and 100°C prior to SDS-PAGE (lanes C through G, respectively). Lane H, Sarkosyl-insoluble OMP preparation. Lane A, molecular size standards as described in the text.

A B C D E F G H

200

97.4 —

68 —

43 —

29 —

18.4 —

14.3 —

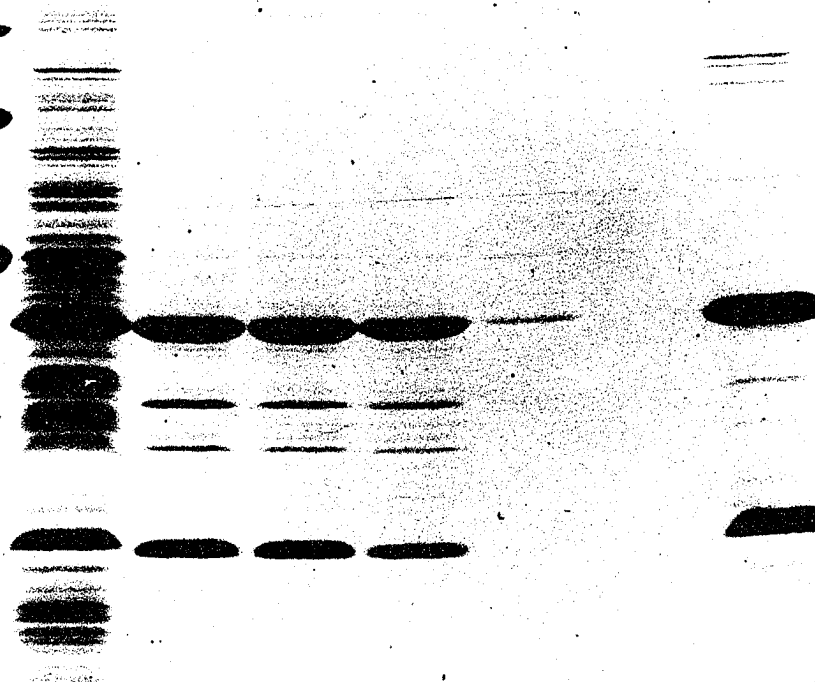
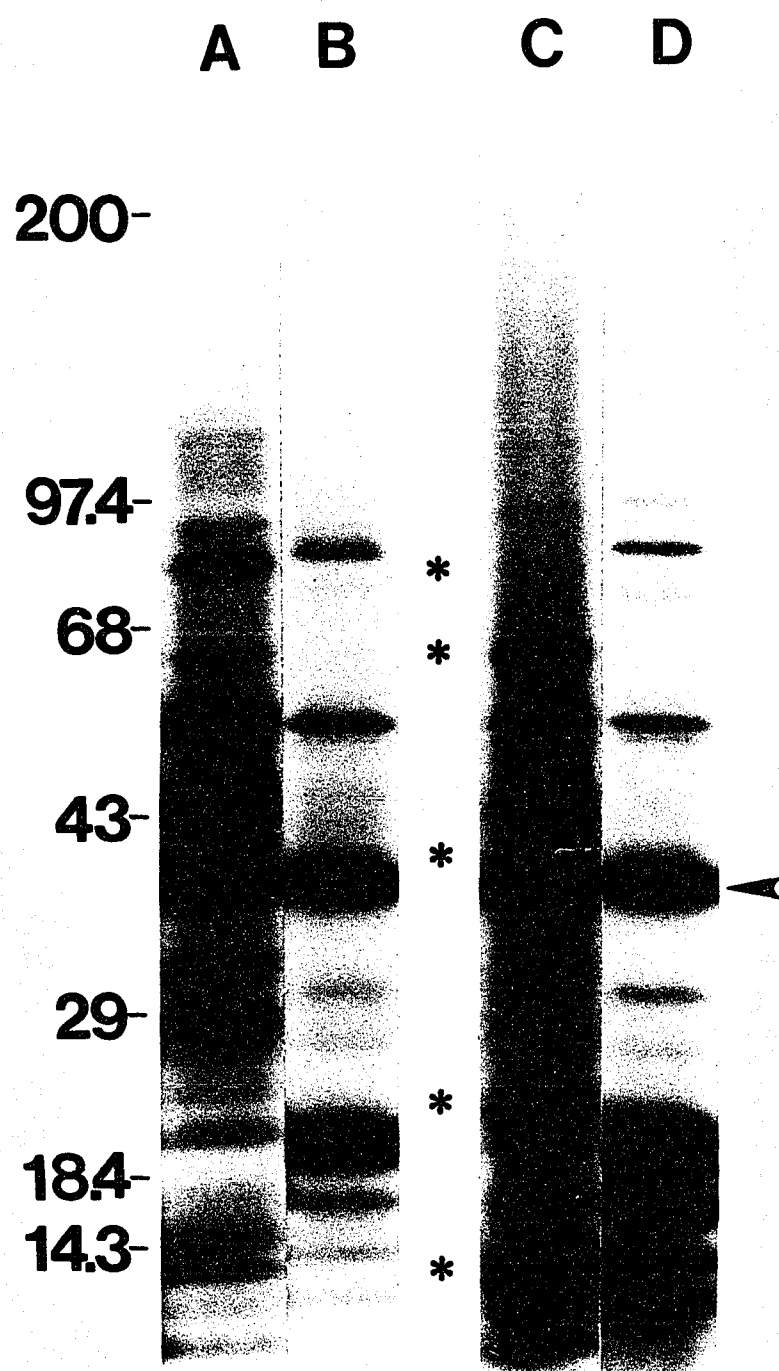


Figure 4. SDS-PAGE analysis of radioiodinated whole cells and outer membranes of B. avium 75. Samples were radioiodinated with Iodo-beads as the catalyst and subjected to electrophoresis after solubilization in sample buffer at 100°C for 5 min. After electrophoresis the gel was exposed to X-ray film. Lanes A and C, ¹²⁵I-labeled whole cells grown on BHIA and BHIB, respectively. Lanes B and D, labeled Sarkosyl-insoluble extracts of cell envelopes from cells grown on BHIA and BHIB, respectively. Asterisks indicate bands observed in surface-labeled whole cells but not in labeled outer membrane preparations. The arrow indicates the major 37K OMP. The positions of the molecular size standards are indicated on the left.



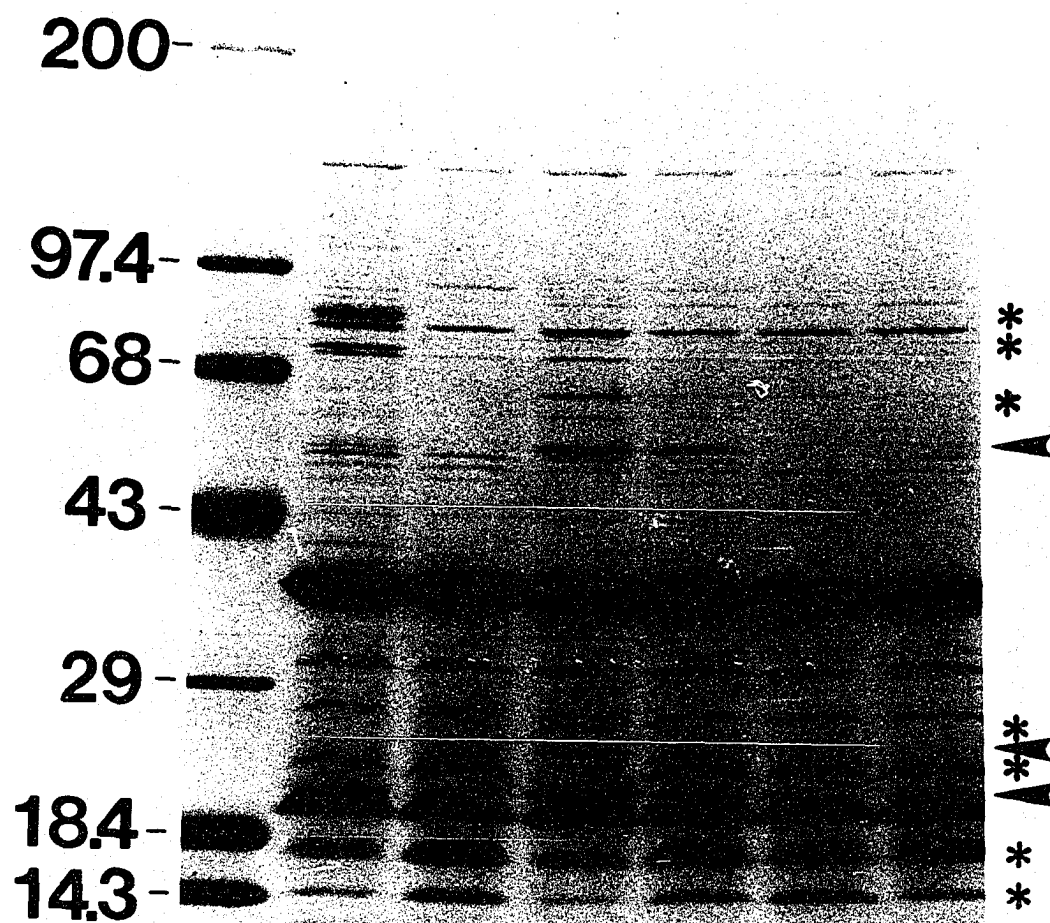
on BHIB. The same bands from BHIB-grown cells were observed to be more prominent in the Coomassie blue-stained gel which indicated the increased intensity of the lower-molecular-weight radiolabeled bands was probably due to an increased amount of protein (Figure 5). If the X-ray film was overexposed, the 41K, 43K, and 143K OMP bands could also be observed in the radiolabeled OMP preparation (data not shown). The most prominent surface-radiolabeled band of whole cells grown on both BHIA and BHIB corresponded to the 37K major OMP (indicated by an arrow in Figure 4). In contrast, the major 21K OMP was poorly surface-labeled in whole cells grown in both media (Figure 4, lanes A and C). Surface-labeled bands that corresponded to other OMPs included the 13.5K, 15K, 18K, 41K, 43K, and 54K bands while the 27K and 31K OMP bands comigrated with two areas of diffuse bands from the labeled whole cells. Bands present in surface-labeled whole cells that did not correspond to OMPs included the 14K, 22K, 39K, 64K, and 79K bands (indicated by asterisks in Figure 4). Profiles of surface-labeled proteins were comparable between cells grown on both media with one exception, a 79K band was observed only in cells grown on BHIA.

Effect of Growth Conditions on Expression of B. avium OMPs

The OMPs isolated from B. avium strain 75 grown on various media were compared to determine the effect of different growth conditions on OMP expression (Figure 5). The major 21K and 37K OMPs were the most prominent bands observed and were present in relatively the same

Figure 5. SDS-PAGE profiles of Sarkosyl-insoluble OMPs isolated from B. avium 75 grown for 36 h at 37°C on the following media: lane B, BHIA; lane C, BHIB; lane D, BGA; lane E, SSM; lane F, SSM containing 20 mM MgSO₄; lane G, SSM containing 500 ug of nicotinic acid per ml. Ten micrograms of protein was applied to each lane. Asterisks indicate OMPs which appear to differ in intensity due to grown in different media. Arrows show the 22K, 24K, and 56K proteins which are apparent in cells grown on BGA and SSM but not in SSM supplemented with MgSO₄ or nicotinic acid. Lane A, molecular size standards shown in kilodaltons are described in text.

A B C D E F G



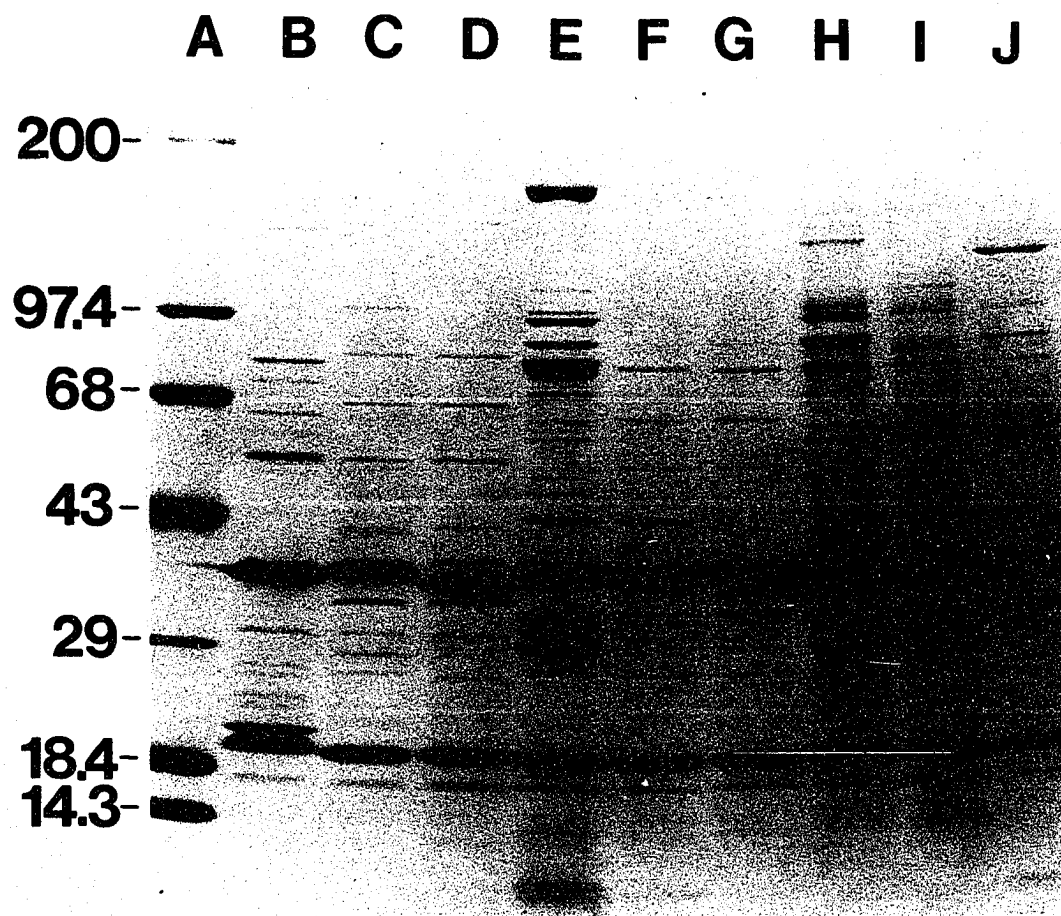
concentration in profiles from cells grown in all media. At least seven minor bands were observed to differ in intensity due to growth in various media (indicated by asterisks in Figure 5). In addition, 22K and 56K bands were prominent in the OMP profile from cells grown on BGA (Figure 5, lane D). The 22K and 56K proteins were present but less intense in OMP profiles from cells grown in SSM and were not apparent in profiles from cells grown in SSM containing nicotinic acid or magnesium sulfate (Figure 5, lanes E to G). The 24K band was present as a minor band in profiles from cells grown on BGA and SSM but was not observed in profiles from cells grown in SSM with nicotinic acid or magnesium sulfate. No other observable differences in Sarkosyl-insoluble protein profiles were noted between cells grown in SSM and in modified SSM.

The effect of incubation time on OMP expression was examined using B. avium 75. Sarkosyl-insoluble protein profiles from cells grown on BHIA and in BHIB for 24 h, 36 h, and 48 h showed no apparent differences due to incubation time (data not shown).

Comparison of Sarkosyl-insoluble Protein Profiles from Bordetella spp.

Sarkosyl-insoluble proteins of Bordetella spp., including B. avium-like and B. bronchiseptica isolates from turkeys with upper respiratory disease, were compared to OMPs of B. avium by SDS-PAGE. All strains were passaged twice on BGA prior to isolation of OMPs. A single major band ranging from 36K to 40K was observed in OMP profiles isolated from each species (Figure 6). A second major OMP band with similar mobility

Figure 6. SDS-PAGE analysis of Sarkosyl-insoluble OMPs from Bordetella spp. grown on BGA. Lane B, B. avium 75; lane C, B. avium-like 023; lane D, B. avium-like 101; lane E, B. bronchiseptica 103; lane F, B. bronchiseptica 469; lane G, B. bronchiseptica Bailey; lane H, B. pertussis 536; lane I, B. pertussis 537, lane J, B. parapertussis 504. Lane A, molecular size standards as described in the text.



as the 21K band of B. avium was present in each lane. A minor 18K band was present in all species but comparison of the remaining bands in the B. avium OMP profile with bands from other species showed few similarities.

The 20 B. avium-like isolates examined could be separated into two groups based on different OMP profiles. The first group contained nine isolates with a major 38K band and was represented by strain 023, while the remaining isolates formed a second group with a major 36K band and were represented by B. avium-like 101 (Figure 6, lanes C and D). Despite the difference in the 36K and 38K bands, the majority of the minor bands and the major 21K band from the two groups had identical mobility. No differences in colonial morphology and standard laboratory tests used to identify the organism were seen between the two groups of B. avium-like isolates (data not shown). Though certain bands in B. avium-like strains 023 and 101 and B. avium 75 OMP profiles had similar or identical mobility, the profiles were distinctly different (Figure 6).

The OMP profiles of B. pertussis, B. parapertussis, and B. bronchiseptica strains resembled those that have been previously described (3, 14, 39). The B. bronchiseptica isolates from turkeys formed an extremely homogeneous group based on Sarkosyl-insoluble OMPs of which the profile of strain 103 is representative (Figure 6, lane E). The B. bronchiseptica 103 profile was clearly different from B. avium and B. avium-like profiles but possessed certain major OMP bands that comigrated with bands in profiles from B. bronchiseptica strains Bailey

and 469 and B. pertussis 536 (Figure 6). As with the other Bordetella spp., strain 103 had major bands in the 21K and 38K range. Major 30K, 32K, 91K, and 100K bands were present in both strain 103 and B. pertussis 536. Certain other bands were seen in common between profiles from strain 103 and the B. bronchiseptica, B. pertussis, and B. parapertussis isolates in Figure 6. In contrast, a major 174K band in the strain 103 profile appeared to be unique among the bordetellae.

DISCUSSION

The OMP-enriched fractions from B. avium isolates show strikingly similar profiles by SDS-PAGE. This result is in agreement with previous studies involving total cellular proteins (23), soluble cell proteins (27), cell envelope proteins (26), and OMPs from a limited number of B. avium strains (22) which have indicated little difference in protein profiles between isolates. Homogeneity of OMP profiles within a species appears to be characteristic of members of the genus Bordetella (33, 35, 39). In certain gram-negative bacteria, OMP profiles differ according to serotypes or biotypes (9, 34) but homogeneity of cell envelope proteins is seen in B. pertussis, even in strains from different serotypes (3). Serotypes have been shown to exist in B. avium (27), unfortunately, little is known about them and a serotyping scheme is not available. By including numerous isolates from geographically diverse areas it is hoped that a representative cross-section of B. avium strains has been examined.

The OMP profile of B. avium appears typical of gram-negative bacteria with a limited number of major bands and numerous minor or less-intensely stained bands (11, 31). The major 21K and 37K OMPs had identical electrophoretic mobility in all isolates examined. Major OMP bands with comparable mobilities were present in other Bordetella species; however, other similarities with B. avium OMP profiles were limited. This result was not unexpected since B. avium has been shown to be relatively distinct genetically and phenotypically from B.

pertussis, B. parapertussis, and B. bronchiseptica (27). The turkey isolates of B. bronchiseptica had OMP profiles that were different from B. avium profiles. The OMP profile of B. bronchiseptica 103 appeared most similar to the B. pertussis 536 profile but certain bands in the turkey isolate were distinctly different from all Bordetella spp.

Initial comparison of Triton X-100-insoluble OMP profiles of B. avium with a B. avium-like isolate showed numerous differences (22). In this study, we demonstrated that Sarkosyl-insoluble protein profiles were distinct between the two groups of organisms. These differences in OMP profiles support the proposal by Jackwood et al. that B. avium and B. avium-like isolates might be different species (25). However, more phenotypic and genetic analysis is needed to determine the taxonomic relationship of the two organisms. Two OMP profile types were observed in B. avium-like organisms. Bordetella avium-like isolates have been shown to revert between two colony types during in vitro growth (12, 26); however, colony type did not appear to correlate with the OMP pattern (data not shown).

Heat-modifiable proteins were observed in OMP profiles from B. avium. OMPs that vary in apparent molecular weight due to changes in solubilization temperature prior to SDS-PAGE are found in many bacteria including Escherichia coli (32), Pseudomonas aeruginosa (20), Vibrio cholerae (38), Actinobacillus pleuropneumoniae (34), and B. pertussis (2, 33). The major 21K and 37K bands and certain minor bands were observed to decrease in apparent molecular weight as solubilization temperatures increased. Incubation at temperatures in excess of 70°C is

frequently required to dissociate protein-protein complexes found in the outer membrane and allow migration of solubilized proteins at a lower molecular weight (31). The noncovalent association of the 21K and 37K OMPs with the peptidoglycan might also affect the heat-modifiability of these major proteins. The 27K and 31K bands were not observed until the OMP preparation was solubilized in sample buffer at 100°C and increased in intensity when the solubilization time at 100°C was lengthened from 5 min to 30 min. It is possible that strong intramolecular or intermolecular associations of these OMPs inhibits conversion to the heat-modified form, requiring extended solubilization times at high temperatures (31). As observed for the major 21K and 37K OMPs, the 27K and 31K proteins were peptidoglycan-associated, requiring temperatures of greater than 60°C in 2% SDS to be released from the murein. Certain OMPs of B. pertussis have been demonstrated to migrate as higher-molecular-weight species at higher solubilization temperatures (2, 33) but proteins with similar characteristics were not seen in B. avium.

Surface-exposed proteins of B. avium were identified by radiolabeling whole cells with ^{125}I . The most prominent bands in the labeled whole cells had molecular weights ranging from 37,000 to 64,000 in bacteria grown on BHIB and from 37,000 to 79,000 in BHIA-grown cells. A band corresponding to the major 37K OMP routinely was the predominant surface-labeled protein in all preparations. This is not surprising since large amounts of the 37K protein in Sarkosyl-insoluble preparations indicate that the protein is most likely a prevalent component of the outer membrane. In contrast, the major 21K protein was

poorly radiolabeled in both whole cell preparations. This might indicate that the 21K protein is not present on the cell surface or that the protein contains no exposed tyrosine residues. Amino acid analysis has shown that the 21K protein contains tyrosine residues but it is possible that these amino acids are buried and not accessible to radioiodination (as described in Section II). Sarkosyl extraction of the total envelope fraction may expose the tyrosine residues of the 21K protein because the protein is strongly labeled in the OMP-enriched fractions. The majority of bands from the radiolabeled whole cells corresponded to bands in the labeled OMP preparations with the exception of five bands (indicated by asterisks in Figure 4). The five bands may represent proteins in the outer membrane which are solubilized during Sarkosyl extraction and therefore are not present in the OMP profiles. The bands, especially the minor bands, could correspond to proteins which are secreted by the whole cells or are exposed to radioiodination due to release by any lysed cells in the whole cell preparations (38).

Comparison of OMP profiles from strain 75 grown on different media indicated that expression of the major 21K and 37K OMPs is constitutive, at least on the media examined. It is not known if the 21K and 37K OMPs are expressed in vivo but antibodies to OMPs with similar molecular weights reportedly are present in sera from experimentally infected turkeys (21).

Expression of certain OMPs (3, 14, 33, 35, 39, 47) and other virulence-associated factors (40, 47) of B. pertussis is affected by two forms of regulation, antigenic modulation and phase variation.

Antigenic modulation is a coordinate, reversible phenotypic change seen when B. pertussis is grown in media containing certain ions and nutrients or at lower temperatures (28). Phase variation results in similar phenotypic changes but is due to a metastable frameshift mutation in the bvg or vir locus (46, 47). The bvg locus encodes proteins which sense environmental stimuli and coordinately regulate transcription of the virulence-associated genes including certain OMP genes of B. pertussis (1). Similar bvg loci have been demonstrated in B. bronchiseptica and B. parapertussis (18) but it is not known if a similar system exists in B. avium. Production of the dermonecrotic toxin of B. avium was reduced by growth in media containing 20 mM MgSO_4 or 500 ug of nicotinic acid per ml, two nutrients that cause phenotypic modulation in B. pertussis (16). Growth at a reduced temperature decreased hemagglutination and reduced expression of many OMPs (22). Recently, spontaneous phase variants of B. avium were isolated which did not express the dermonecrotic toxin and four OMPs (15). We have demonstrated variable expression of at least 10 B. avium OMPs caused by growth in different media. Expression of the 22K, 24K, and 56K OMPs was reduced when strain 75 was grown under modulating conditions (i.e., in media containing MgSO_4 or nicotinic acid). This indicates control of expression of certain B. avium OMPs could be similar to that in other members of the genus Bordetella and provides more evidence that a coordinately-regulated system related to the bvg system might be present in B. avium.

The major 37K OMP of B. avium shares certain characteristics with

the major 40K OMP of B. pertussis which functions as an anion-selective porin (4). The proteins had similar mobility in SDS-PAGE and the 37K and 40K proteins were the most prominent bands in Sarkosyl-insoluble OMP profiles from B. avium and B. pertussis, respectively. The 40K protein has been shown to be constitutively expressed in several B. pertussis strains grown on different media and in phenotypically-modulated and avirulent-phase cells (3, 14). Further characterization by several investigators has shown that the 40K protein is exposed to surface-radioiodination (2, 3, 35), is heat-modifiable, is not affected by the reducing agent 2-ME, and is noncovalently associated with the peptidoglycan layer (2). Though we have shown that the 37K OMP of B. avium possesses these characteristics, we have not attempted to directly compare the two OMPs and more work is needed to determine the structure and function of the major 37K OMP of B. avium and its relationship with the 40K porin protein of B. pertussis, if any.

We have characterized the proteins in the outer membrane of B. avium and have compared them with OMPs from other Bordetella spp. It is likely that many of the OMPs of B. avium described are in fact true OMPs because they share characteristics with OMPs of other gram-negative bacteria such as Sarkosyl-insolubility, surface-exposure to radioiodination, heat-modifiability, and noncovalent association with the peptidoglycan layer. More work is needed to further characterize the OMPs of B. avium and their involvement in the pathogenesis of turkey bordetellosis. We have shown that expression of certain OMPs depends on in vitro growth conditions. It is essential that the differences in OMP

expression be taken into consideration in further experimental studies and in vaccine production because antigenicity of the organism may be affected by these changes.

LITERATURE CITED

1. Arico, B., J. F. Miller, C. Roy, S. Stibitz, D. Monack, S. Falkow, R. Gross, and R. Rappuoli. 1989. Sequences required for expression of Bordetella pertussis virulence factors share homology with prokaryotic signal transduction proteins. Proc. Natl. Acad. Sci. USA 86:6671-6675.
2. Armstrong, S. K., and C. D. Parker. 1986. Heat-modifiable envelope proteins of Bordetella pertussis. Infect. Immun. 54:109-117.
3. Armstrong, S. K., and C. D. Parker. 1986. Surface proteins of Bordetella pertussis: Comparison of virulent and avirulent strains and effects of phenotypic modulation. Infect. Immun. 54:308-314.
4. Armstrong, S. K., T. R. Parr, Jr., C. D. Parker, and R. E. W. Hancock. 1986. Bordetella pertussis major outer membrane porin protein forms small, anion-selective channels in lipid bilayer membranes. J. Bacteriol. 166:212-216.
5. Arp, L. H., and N. F. Cheville. 1984. Tracheal lesions in young turkeys infected with Bordetella avium. Am. J. Vet. Res. 45:2196-2200.
6. Arp, L. H., and J. A. Fagerland. 1987. Ultrastructural pathology of Bordetella avium infection in turkeys. Vet. Pathol. 24:411-418.
7. Arp, L. H., and D. H. Hellwig. 1988. Passive immunization versus adhesion of Bordetella avium to the tracheal mucosa of turkeys. Avian Dis. 32:494-450.
8. Arp, L. H., R. D. Leyh, and R. W. Griffith. 1988. Adherence of Bordetella avium to tracheal mucosa of turkeys: Correlation with hemagglutination. Am. J. Vet. Res. 49:693-696.
9. Barenkamp, S. J., R. S. Munson, Jr., and Dan M. Granoff. 1981. Subtyping isolates of Haemophilus influenzae type b by outer-membrane protein profiles. J. Infect. Dis. 143:668-676.
10. Barnes, H. J., and M. S. Hofstad. 1983. Susceptibility of turkey poults from vaccinated and unvaccinated hens to alcaligenes rhinotracheitis (turkey coryza). Avian Dis. 27:378-392.

11. Benz, R. 1988. Structure and function of porins from gram-negative bacteria. *Ann. Rev. Microbiol.* 42:359-93.
12. Berkhoff, H. A., and G. D. Riddle. 1984. Differentiation of Alcaligenes-like bacteria of avian origin and comparison with Alcaligenes spp. reference strains. *J. Clin. Microbiol.* 19:477-481.
13. Burke, D. S., and M. M. Jensen. 1980. Immunization against turkey coryza by colonization with mutants of Alcaligenes faecalis. *Avian Dis.* 24:726-733.
14. Ezzel, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark. 1981. Phase-shift markers in Bordetella: Alterations in envelope proteins. *J. Infect. Dis.* 143:562-569.
15. Gentry-Weeks, C., and D. L. Provence. 1990. Isolation and characterization of phase variants of Bordetella avium. Abstract B-147. 90th Ann. Meet. Am. Soc. Microbiol., Anaheim.
16. Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss III. 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of Bordetella avium. *Infect. Immun.* 56:1698-1707.
17. Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons. 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an Alcaligenes faecalis isolate. *Infect. Immun.* 42:350-355.
18. Gross, R., and R. Rappuoli. 1988. Positive regulation of pertussis toxin expression. *Proc. Natl. Acad. Sci. USA* 85:3913-3917.
19. Hames, B. D. 1981. An introduction to polyacrylamide gel electrophoresis. Pages 1-91 in B. D. Hames, and D. Rickwood, eds. *Gel electrophoresis of proteins*. IRL Press, Oxford.
20. Hancock, R. E. W., and A. M. Carey. 1979. Outer membrane of Pseudomonas aeruginosa: Heat- and 2-mercaptoethanol-modifiable proteins. *J. Bacteriol.* 140:902-910.
21. Hellwig, D. H., and L. H. Arp. 1990. Identification of Bordetella avium antigens recognized after experimental inoculation in turkeys. *Am. J. Vet. Res.* 51:1188-1191.

22. Hellwig, D. H., L. H. Arp, and J. A. Fagerland. 1988. A comparison of outer membrane proteins and surface characteristics of adhesive and non-adhesive phenotypes of Bordetella avium. Avian Dis. 32:787-792.
23. Hinz, K. -H., G. Glunder, and K. J. Romer. 1983. A comparative study of avian Bordetella-like strains, Bordetella bronchiseptica, Alcaligenes faecalis and other related nonfermentatable bacteria. Avian Pathol. 12:263-276.
24. Jackwood, M. W., and Y. M. Saif. 1987. Pili of Bordetella avium: Expression, characterization, and role in adherence. Avian Dis. 31:277-286.
25. Jackwood, M. W., M. Sasser, and Y. M. Saif. 1986. Contribution to the taxonomy of the turkey coryza agent: Cellular fatty acid analysis of the bacterium. Avian Dis. 30:172-178.
26. Jackwood, M. W., Y. M. Saif, P. D. Moorhead, and R. N. Dearth. 1985. Further characterization of the agent causing coryza in turkey. Avian Dis. 29:690-705.
27. Kersters, K., K. -H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley. 1984. Bordetella avium sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int. J. Syst. Bacteriol. 34:56-70.
28. Lacey, B. W. 1960. Antigenic modulation of Bordetella pertussis. J. Hyg. 58:57-93.
29. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
30. Luginbuhl, G. H., J. M. Rader, and D. G. Simmons. 1984. Evidence for plasmid DNA in Alcaligenes faecalis. Am. J. Vet. Res. 45:1679-1681.
31. Lugtenberg, B., and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. Biochim. Biophys. Acta 737:51-115.
32. Nakamura, K., and S. Mizushima. 1976. Effects of heating in dodecyl sulfate solution on the conformation and electrophoretic mobility of isolated major outer membrane proteins from Escherichia coli K-12. J. Biochem. 80:1411-1422.
33. Parton, R., and A. C. Wardlaw. 1975. Cell-envelope proteins of Bordetella pertussis. J. Med. Microbiol. 8:47-57.

34. Rapp, V. J., R. S. Munson, Jr., and R. F. Ross. 1986. Outer membrane protein profiles of Haemophilus pleuropneumoniae. Infect. Immun. 52:414-420.
35. Redhead, K. 1983. Variability in the surface exposure of the outer membrane proteins of Bordetella pertussis. FEMS Microbiol. Lett. 17:35-39.
36. Redinbaugh, M. G., and R. B. Turley. 1986. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. 153:267-271.
37. Relman, D. A., M. Domenighini, E. Tuomanen, R. Rappuoli, and S. Falkow. 1989. Filamentous hemagglutinin of Bordetella pertussis: Nucleotide sequence and crucial role in adherence. Proc. Natl. Acad. Sci. USA 86:2637-2641.
38. Richardson, K., and C. D. Parker. 1985. Identification and characterization of Vibrio cholerae surface proteins by radioiodination. Infect. Immun. 48:87-93.
39. Robinson, A., and D. C. Hawkins. 1983. Structure and biological properties of solubilized envelope proteins of Bordetella pertussis. Infect. Immun. 39:590-598.
40. Robinson, A., C. J. Duggleby, A. R. Gorringe, and I. Livey. 1987. Antigenic variation in Bordetella pertussis. Pages 147-161 in M. A. Horwitz, ed. Bacteria-host cell interaction. Alan R. Liss, Inc., New York.
41. Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. G. Jackwood. 1980. Observations on Alcaligenes faecalis infection in turkeys. Avian Dis. 24:665-684.
42. Schneider, D. R., and C. D. Parker. 1982. Effect of pyridines on phenotypic properties of Bordetella pertussis. Infect. Immun. 38:548-553.
43. Simmons, D. G., L. P. Rose, K. A. Brogden, and R. B. Rimler. 1984. Partial characterization of the hemagglutinin of Alcaligenes faecalis. Avian Dis. 28:700-709.
44. Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller. 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. 23:194-203.

45. Slavik, M. F., J. K. Skeeles, J. N. Beasley, G. C. Harris, P. Roblee, and D. Hellwig. 1981. Effect of humidity on infection of turkeys with Alcaligenes faecalis. Avian Dis. 25:936-942.
46. Stibtz, S., W. Aaronson, D. Monack, and S. Falkow. 1989. Phase variation in Bordetella pertussis by frameshift mutation in a gene for a novel two-component system. Nature 338:266-269.
47. Weiss, A. A., and S. Falkow. 1984. Genetic analysis of phase change in Bordetella pertussis. Infect. Immun. 43:263-269.

SECTION II. CHARACTERIZATION OF THE 21-KILODALTON OUTER MEMBRANE
PROTEIN OF BORDETELLA AVIUM

SUMMARY

The amino acid composition and amino-terminal sequence of the major 21,000-molecular-weight outer membrane protein of Bordetella avium were determined. Proteins from a Sarkosyl-insoluble outer membrane protein-enriched fraction were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto a polyvinylidene difluoride membrane. The membrane was stained for protein visualization and a band containing the major outer membrane protein was excised and used for amino acid analysis and amino-terminal sequencing. Aspartic acid and asparagine, glutamic acid and glutamine, and alanine were the most prevalent amino acids in the protein. A limited amino-terminal sequence of QTVDN was obtained. Protein instability or partial blockage of the amino-terminal residue limited sequence determination to five amino acids.

INTRODUCTION

Bordetella avium causes turkey bordetellosis, an upper respiratory disease in poults which is characterized by oculonasal discharge, sneezing, reduced weight gain, tracheal collapse, and death in severe cases (1, 6, 19, 20, 22). Like other members of the genus Bordetella, B. avium exhibits a tropism for the respiratory tract epithelium (1, 2). Bacteria have been shown to be present on the tracheal mucosa in microcolonies which are in intimate contact with the ciliated epithelium. Colonization results in inflammation with loss of epithelium and distortion of mucosa and tracheal rings. Because of the direct interaction of the bacterial surface with epithelial and immune cells of the host, surface structures of B. avium might play an important role in colonization of the trachea and subsequent immunity.

The cell surface of B. avium appears typical for gram-negative bacteria. Proteinaceous surface appendages include peritrichous flagella and fimbriae (pili) (11, 13). A hemagglutinin is present in virulent strains (12, 21) and, along with the fimbriae, may be involved in adherence to respiratory epithelium (4, 11). Outer membrane proteins (OMPs) of B. avium have been examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The OMP profiles from all isolates are similar and possess two major protein bands with approximate molecular weights of 21,000 and 37,000 (21K and 37K proteins) and numerous minor proteins (8, 15). The humoral immune response to the OMPs has been characterized in experimentally infected

turkeys (3, 7). Using Western immunoblot analysis, Hellwig and Arp have shown that antibodies in sera and tracheal washings from turkeys with bordetellosis recognized at least eight antigens in an OMP-enriched extract of B. avium (7). Antibody reactivity was especially intense to the major 21K OMP in both sera and tracheal washings.

Because the 21K OMP of B. avium has been shown to elicit a strong local and systemic humoral immune response in experimentally infected turkeys, further characterization of the protein may help in understanding the pathogenesis of bordetellosis. In this paper, we describe the initial chemical characterization of the major 21K protein of B. avium. The protein was purified by SDS-PAGE and used for determination of the amino acid composition and the amino-terminal amino acid sequence.

MATERIALS AND METHODS

Bacterial Strain

Bordetella avium 75 was a virulent isolate originally cultured from a 3-week-old turkey with bordetellosis in Iowa (1). The isolate was maintained at -80°C in a 20% glycerin/80% brain heart infusion (BHI, Difco Laboratories, Detroit, Mich.) broth mixture during the course of the study.

Media and Growth Conditions

Bordetella avium 75 was grown on BHI agar (BHIA) containing 1.5% agar (BiTek agar, Difco) at 37°C for 36 h, transferred to BHIA or BHI broth (BHIB) and incubated at 37°C for 36 h. The BHIB was incubated at 210 rpm in a shaking incubator (model G25-KC; New Brunswick Scientific Co., Inc., Edison, N. J.). Strain 75 was also grown on Bordet-Gengou agar (Difco) containing 15% defibrinated sheep blood and 1% glycerol (BGA) for 36 h at 37°C. Bacteria were harvested from plates in 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid; pH 7.4) buffer (Sigma Chemical Co., St. Louis, Mo.) or from broth and collected by centrifugation at 10,000 x g for 20 min. The bacteria were washed twice in 10 mM HEPES (pH 7.4) buffer and approximately 1.5 gm (wet weight) of cells were suspended in 15 ml of buffer and stored at -80°C. The cells were used within two weeks for isolation of OMPs.

OMP Preparation

The OMP-enriched fractions were prepared by a modification of the procedure of Hellwig and Arp (7). Briefly, the cells were thawed and the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to a final concentration of 0.1 mM. The cells were disrupted by a sonicator (model 350; Branson Sonic Power Co., Danbury, Conn.) for 10, one minute bursts (50% cycle, power setting 7) while cooling in an ice water bath. Whole cells and large debris were removed by centrifugation at 5,000 x g for 20 min and the total membrane fraction was collected from the supernatant by centrifugation at 100,000 x g for 60 min at 4°C. The membrane fraction was suspended in 1% (wt/vol) Sarkosyl (sodium lauryl sarcosine; International Biotechnologies, Inc., New Haven, Conn.) in 10 mM HEPES (pH 7.4) buffer containing 0.1 mM PMSF for 30 min at room temperature. The detergent-insoluble material containing the OMP-enriched fraction was collected by centrifugation at 100,000 x g for 60 min at 4°C, suspended in deionized water to a concentration of 8.0 mg of protein per ml, and stored at -80°C. The protein concentration was determined by the bicinchoninic acid assay (18; Pierce Chemical Company, Rockford, Ill.) using bovine serum albumin as a standard.

SDS-PAGE

A modified Laemmli (14) procedure was used to separate the detergent-insoluble proteins. Electrophoresis was carried out on a discontinuous gel consisting of a 4% stacking gel and a 6 to 20%

gradient resolving gel in a 16 cm vertical slab apparatus (Protean II; Bio-Rad Laboratories, Richmond, Calif.) following the procedure in the suppliers catalog. Proteins were solubilized in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, 20% glycerol, 0.001% bromophenol blue, and 0.0625 M Tris hydrochloride (pH 6.8) at 100°C for 5 min. The gel was loaded with 10 ug of protein per lane and electrophoresis was carried out at constant current of 25 mA through the stacking gel and 35 mA until the dye front was approximately 1 cm from the bottom of the gel. Molecular size standards described in Figure 1 were used (Life Technologies, Inc., Gaithersburg, Md.). The gel was stained with Coomassie brilliant blue R250 for visualization of the bands.

Amino Acid Analysis and Microsequencing

The Sarkosyl-insoluble OMP-enriched preparation from strain 75 (150 ug of protein per lane) grown in BHIB was separated by SDS-PAGE using a 6 to 20% gradient gel as described above. Following electrophoresis the gel was soaked in transfer buffer containing 10mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid; pH 11.0)(Sigma) in 10% methanol for 15 min (17). The proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore Corporation, Bedford Ma.) in a transblot cell (Bio-Rad) at 600 mA for 2 h. The PVDF membrane was washed in deionized water for 5 min, stained with 0.1% Coomassie brilliant blue R250 in 50% methanol for 5 min, and

destained in 50% methanol, 10% acetic acid for 10 min. The membrane was washed with deionized water for 10 min, air-dried, and stored at -20°C until used for amino acid analysis and protein sequencing.

The band containing the 21K OMP was excised and the protein was directly hydrolyzed on the PVDF membrane in 6 N HCl at 150°C for 1 h under vacuum. The membrane was placed on a derivatizer (model 420A; Applied Biosystems, Inc.) and the phenylthiocarbamyl-amino acids were separated on a C-18 reverse-phase high-performance liquid chromatography column. The phenylthiocarbamyl chromophore was detected at 254 nm.

A second band containing approximately 20 ug of the 21K protein was excised from the electroblotted PVDF membrane. The amino-terminal sequence of the 21K protein was determined from this sample using a peptide sequencer (model 477A; Applied Biosystems) coupled to an amino acid analyzer (model 120A; Applied Biosystems).

RESULTS

SDS-PAGE

The Sarkosyl-insoluble OMP-enriched fractions from B. avium strain 75 grown on BHIA, BHIB, and BGA were examined by SDS-PAGE. Major 21K and 37K bands as well as at least 11 less-intensely stained bands were observed on the Coomassie blue-stained gel (Figure 1). The OMP profile from cells grown on BGA contained a prominent 22K band that was not completely resolved from the 21K band (Figure 1, lane D). The 22K band was observed at low levels in BHIA-grown cells when the lane was overloaded with protein (data not shown) but was not seen in profiles isolated from cells grown in BHIB. Because of the difficulty in resolving the 21K and 22K proteins in a single-dimension gel, OMP preparations containing no observable 22K protein were isolated from cells grown on BHIB and used for amino acid analysis and amino-terminal sequencing.

Amino Acid Analysis

Amino acid analysis of the 21K OMP from B. avium 75 indicated that aspartic acid and asparagine (13.2%), glutamic acid and glutamine (10.0%), and alanine (11.4%) were the most prevalent amino acids (Table 1). Other amino acids present included: serine, glycine, arginine, threonine, proline, tyrosine, valine, isoleucine, leucine, phenylalanine, and lysine. Analysis indicated a trace of cysteine was

Figure 1. SDS-PAGE of Sarkosyl-insoluble OMP-enriched fractions prepared from B. avium 75. OMP-enriched fractions from strain 75 grown the following media: lane B, BHIA; lane C, BHIB; lane D, BGA. Ten micrograms of protein was applied to each lane. The arrow indicates the 21K OMP. Lane A, molecular size standards. Molecular size standards shown in kilodaltons: myosin (H-chain)(200); phosphorylase B (97.4); bovine serum albumin (68); ovalbumin (43); carbonic anhydrase (29); B-lactoglobulin (18.4); lysozyme (14.3).

A B C D

200-

97.4- —

68- —

43- —

29- —

18.4- —

14.3- —

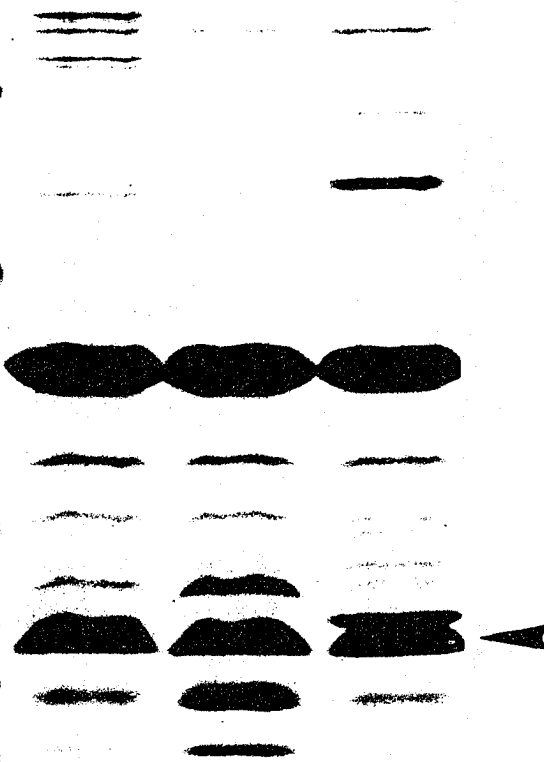


Table 1. Amino acid composition of the 21K OMP of Bordetella avium 75

Amino acid	Number of residues per mol of 21K protein ^a	mol%
Aspartic acid and asparagine	25	13.2
Glutamic acid and glutamine	19	10.0
Serine	8	3.9
Glycine	16	8.7
Arginine	15	7.9
Threonine	12	6.4
Alanine	22	11.4
Proline	11	5.6
Tyrosine	5	2.4
Valine	13	7.1
Cysteine	ND ^b	0.3
Isoleucine	11	5.7
Leucine	10	5.3
Phenylalanine	6	3.3
Lysine	15	7.9

^aEstimated number of residues using the ratio of amino acids determined by amino acid analysis and based on a protein molecular weight of 21,000. Results are rounded to the nearest whole number.

^bNot determinable.

present in the 21K protein while the amino acids histidine and methionine were not detected. No attempt was made to quantitate the tryptophan and cysteine residues.

Amino-terminal Sequence

The amino-terminal sequence of the 21K OMP was determined. The first cycle released a glutamine but in the following cycles the background increased rapidly making further sequence determination difficult. The sequence of the first five amino acids of the amino terminus was QTVDN. No amino acids could be assigned to the sixth and seventh cycles but asparagine was released by the eighth cycle. After eight cycles the sequence could no longer be determined due to background. The amino-terminal sequence analysis was repeated on a second sample of the 21K OMP isolated from a fresh batch of BHIB-grown cells but the result was identical.

DISCUSSION

We have reported on initial chemical characterization of the major 21K OMP of B. avium strain 75. A major protein with an identical molecular weight is present in OMP profiles from all B. avium isolates examined (15); however, it is not known if the 21K OMPs of all B. avium isolates are the same.

Results from amino acid analysis of the 21K OMP are shown in Table 1. No unusual characteristics were noted in the amino acid composition of the protein. The relatively polar nature of the 21K protein is typical of certain OMPs from other gram-negative bacteria (16). Amino acid analysis indicated a trace of cysteine was present but this amino acid is extremely susceptible to oxidation on the PVDF membrane during acid hydrolysis and the exact number of residues can not be determined using this method (23). The presence of cysteine in the 21K protein indicates disulfide bonds might exist in the natural protein. Previous work, however, has demonstrated that the reducing agent 2-mercaptoethanol has no effect on migration of the 21K protein in SDS-PAGE which indicates that intrachain disulfide bonds might not be present (15). The amount of tryptophan in the protein was not determined because the acid used for hydrolysis destroys the indole ring and makes quantitation of this amino acid impossible (23).

Attempts to determine the amino-terminal sequence of the 21K protein were not as successful as hoped. The sequence of the first five residues was determined but background increased rapidly during each

cycle and prevented further amino acid identification except for the eighth residue. The increased background observed could be caused by slow breakdown of the polypeptide in the sequencer or temporary blockage of the amino-terminal end. Inglis has reported that cleavage of the peptide chain can occur at aspartic acid in the presence of dilute acids (10). Aspartic acid is a common amino acid in the 21K OMP and sequencing has indicated that the amino acid is present in the amino-terminal region of the protein. The prevalence of aspartic acid may make the protein particularly susceptible to hydrolysis in the presence of the trifluoroacetic acid used in the sequenator. Blockage of the amino-terminal amino acid can occur during electrophoresis (9). If blockage was temporary and was slowly removed during sequencing, this could contribute to increased background and prevent further sequence determination.

It is clear that more work needs to be done to further characterize the 21K OMP. Gentry-Weeks et al. have recently reported on the cloning of a 21K OMP of B. avium strain 197 and expression of the recombinant protein in Escherichia coli and Salmonella typhimurium (5). It is not known if the two proteins are the same but comparison of the amino acid sequence derived from the nucleotide sequence of the gene with the amino-terminal sequence of the 21K protein should make comparison of the proteins possible.

LITERATURE CITED

1. Arp, L. H., and N. F. Cheville. 1984. Tracheal lesions in young turkeys infected with Bordetella avium. Am. J. Vet. Res. 45:2196-2200.
2. Arp, L. H., and J. A. Fagerland. 1987. Ultrastructural pathology of Bordetella avium infection in turkeys. Vet. Pathol. 24:411-418.
3. Arp, L. H., and D. H. Hellwig. 1988. Passive immunization versus adhesion of Bordetella avium to the tracheal mucosa of turkeys. Avian Dis. 32:494-500.
4. Arp, L. H., R. D. Leyh, and R. W. Griffith. 1988. Adherence of Bordetella avium to tracheal mucosa of turkeys: Correlation with hemagglutination. Am. J. Vet. Res. 49:693-696.
5. Gentry-Weeks, C., A. -L. Hultsch, S. M. Kelly, and R. Curtiss III. 1990. Molecular cloning of outer membrane protein genes of Bordetella avium 197 and expression of a B. avium 21-kDa outer membrane protein in Salmonella typhimurium. Abstract B-148. 90th Ann. Meet. Am. Soc. Microbiol., Anaheim.
6. Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons. 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an Alcaligenes faecalis isolate. Infect. Immun. 42:350-355.
7. Hellwig, D. H., and L. H. Arp. 1990. Identification of Bordetella avium antigens recognized after experimental inoculation in turkeys. Am. J. Vet. Res. 51:1188-1191.
8. Hellwig, D. H., L. H. Arp, and J. A. Fagerland. 1988. A comparison of outer membrane proteins and surface characteristics of adhesive and non-adhesive phenotypes of Bordetella avium. Avian Dis. 32:787-792.
9. Hunkapiller, M. W., E. Lujan, F. Ostrander, and L. E. Hood. 1983. Isolation of microgram quantities of proteins from polyacrylamide gels for amino acid sequence analysis. Methods Enzymol. 91:227-236.
10. Inglis, A. S. 1983. Cleavage at aspartic acid. Methods Enzymol. 91:324-332.

11. Jackwood, M. W., and Y. M. Saif. 1987. Pili of Bordetella avium: Expression, characterization, and role in adherence. Avian Dis. 31:277-286.
12. Jackwood, M. W., Y. M. Saif, P. D. Moorhead, and R. N. Dearth. 1985. Further characterization of the agent causing coryza in turkey. Avian Dis. 29:690-705.
13. Kersters, K., K. -H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley. 1984. Bordetella avium sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int. J. Syst. Bacteriol. 34:56-70.
14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
15. Leyh, R. D., and R. W. Griffith. 1989. Outer membrane protein profiles of Bordetella avium. Abstract 69. 70th Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.
16. Lugtenburg, B. and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. Biochim. Biophys. Acta 737:51-115.
17. Matsudaira, P. 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. 262:10035-10038.
18. Redinbaugh, M. G., and R. B. Turley. 1986. Adaptation of the bicinchoninic acid protein assay for use with microtiter plates and sucrose gradient fractions. Anal. Biochem. 153:267-271.
19. Rimler, R. B., and D. G. Simmons. 1983. Differentiation among bacteria isolated from turkeys with coryza (rhinotracheitis). Avian Dis. 27:491-500.
20. Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. G. Jackwood. 1980. Observations on Alcaligenes faecalis infection in turkeys. Avian Dis. 24:665-684.
21. Simmons, D. G., L. P. Rose, K. A. Brogden, and R. B. Rimler. 1984. Partial characterization of the hemagglutinin of Alcaligenes faecalis. Avian Dis. 28:700-709.
22. Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller. 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. 23:194-203.

23. Zubay, G. 1988. The building blocks of proteins: Amino acids, peptides, and polypeptides. Pages 31-52 in G. Zubay, ed. Biochemistry. Macmillan Publishing Co., New York.

GENERAL SUMMARY AND DISCUSSION

During early phases of the investigation involving characterization of the 21,000-molecular-weight outer membrane protein (21K OMP), it became apparent that information regarding the OMPs of Bordetella avium was limited and a better understanding of these proteins was needed before the research could continue. In the first study presented in this dissertation, an attempt was made to "lay the groundwork" for further research involving B. avium OMPs. Sarkosyl-insoluble OMP-enriched fractions from numerous isolates were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and compared to OMP profiles from other members of the genus Bordetella. The B. avium OMPs were evaluated for heat-modifiability, noncovalent association with the peptidoglycan layer, and surface-exposure to radioiodination. The effect of variations in the growth medium on the expression of the OMPs of B. avium was also examined.

The OMP profiles of 50 B. avium isolates from various geographically diverse areas were examined. To determine if the OMP profiles had changed over time, isolates obtained from turkeys at least six years previously and recent field isolates from turkeys with upper respiratory disease were included in the group. Two major bands representing the 21K and 37K OMPs and at least 13 less-intensely stained bands with molecular weights ranging from 13,500 to 143,000 were apparent in the OMP profiles. All profiles were very similar with only minor differences between certain isolates. These results are not

surprising since phenotypic and genotypic studies have shown that B. avium isolates form a relatively homogeneous species (65, 77). However, homogeneity of the OMP profiles does not insure that the isolates are the same antigenically because in a related organism, B. pertussis, strains from different serotypes have very similar OMP profiles (6). Bordetella avium isolates have been shown to possess both common surface antigens and type-specific antigens (77); however, the relationship of these antigens with the OMPs is not known.

Certain OMPs of B. avium were heat-modifiable, that is, the proteins had lower apparent molecular weights when the solubilization temperature was increased prior to electrophoresis. Heat-modifiability of OMPs is frequently observed in other bacteria and is characteristic of certain classes of OMPs (5, 17, 90, 108). Protein heat-modifiability can result from strong intramolecular interactions or noncovalent interactions with other proteins in the outer membrane or with the underlying peptidoglycan layer. The interactions can prevent the complete solubilization and denaturation of certain OMPs at low solubilization temperatures (90). The major 21K and 37K OMPs of B. avium were heat-modifiable and were noncovalently associated with the peptidoglycan. Two other OMPs, the 27K and 31K proteins, exhibited similar characteristics but required extended solubilization times at 100°C to be totally converted to the heat-modified form.

Exposure of B. avium proteins on the cell surface was determined by radiolabeling whole cells with ¹²⁵I followed by SDS-PAGE. The surface-labeled proteins were compared to radioiodinated outer membrane

preparations and of the 13 bands present in profiles from labeled whole cells, eight corresponded to bands in the radiolabeled outer membranes. The predominant surface-labeled protein corresponded to the major 37K OMP. This is not surprising since the 37K protein is the most prevalent protein in the OMP profile. Interestingly, the major 21K OMP was poorly labeled by surface-radioiodination. This indicates the 21K protein is either not exposed on the cell surface or contains no exposed tyrosine residues. As indicated by amino acid analysis, tyrosine is present in the 21K OMP but it is possible that the residues are inaccessible to radioiodination. Sarkosyl extraction of the cell envelope appears to expose the protein to labeling since the 21K band is prevalent in the labeled outer membrane preparations. The 21K OMP appears to induce a particularly intense antibody response in turkeys experimentally infected with B. avium (60). This indicates that the 21K OMP is accessible to cells of the immune system and suggests that it is exposed on the cell surface.

There has been some confusion regarding the relationship of virulent B. avium and avirulent B. avium-like organisms. Both organisms are frequently isolated from turkeys with upper respiratory disease (73, 116). The organisms are phenotypically similar, with the most striking distinction between the two groups being the difference in virulence. The many differences observed in this study between the OMP profiles of B. avium and B. avium-like organisms support the proposal of Jackwood et al. that the two groups of bacteria represent different species (72). The B. avium OMP profile was also distinct from profiles of

representative isolates of B. bronchiseptica, B. pertussis, and B. parapertussis.

Bordetella bronchiseptica is frequently isolated from the upper respiratory tract of turkeys (72, 73, 116). These organisms have been found to be avirulent in experimentally infected turkeys. In this study, examination of 16 B. bronchiseptica isolates from turkeys indicated the bacteria form a homogeneous group based on OMP profiles. The OMP profile of a representative turkey isolate of B. bronchiseptica shared certain bands with the virulent B. bronchiseptica isolates from humans and swine but was most similar to the B. pertussis 536 profile.

Expression of certain OMPs and the majority of virulence-associated factors in B. pertussis is affected by two related forms of coordinate regulation, antigenic or phenotypic modulation and phase variation (6, 40, 82, 148). Antigenic modulation and phase variation also occur in B. bronchiseptica and B. parapertussis (40, 68, 85, 109). Recently, both forms of regulation have been described in B. avium in genes encoding the dermonecrotic toxin and in certain OMP genes (47, 49). In this study, expression of at least eight OMPs appeared to be affected by growth of B. avium in different media. Interestingly, expression of the 22K, 24K, and 56K OMPs was reduced under conditions that induce antigenic modulation in the other Bordetella spp. This provides more evidence that a coordinately regulated system related to that found in B. pertussis, B. parapertussis, and B. bronchiseptica might be present in B. avium.

In the second study, the 21K OMP of B. avium was partially purified

by SDS-PAGE and the amino acid composition and amino-terminal amino acid sequence was determined. No unusual characteristics were noted in the amino acid composition of the 21K OMP. The relatively polar nature of the protein is typical of many OMPs of other gram-negative bacteria (90).

Repeated attempts to determine the amino-terminal amino acid sequence met with limited success. The rapid increase in background during each cycle restricted amino acid identification to the first five and the eighth residues. Recently, Gentry-Weeks et al. have reported the cloning of a 21K OMP gene of B. avium and expression of the recombinant protein in Escherichia coli and Salmonella typhimurium (48). It is not known if this protein is the same as the major 21K OMP examined in this study but comparison of the amino acid sequence derived from the nucleotide sequence of the cloned gene with the amino-terminal sequence of the 21K OMP reported in this study should help to determine if the proteins are the same.

It is likely that many of the B. avium OMPs described in this study are in fact true OMPs because they share characteristics with OMPs of other bacteria such as Sarkosyl-insolubility, heat-modifiability, cell surface-exposure, and noncovalent association with the peptidoglycan layer. Because proteins make up nearly half of the total mass of the outer membrane, they are an important component of the interface between the bacterium and its surrounding environment. Characterization of the OMPs of B. avium should lead to a better understanding of the pathogenesis of turkey bordetellosis but more work is needed to further

evaluate the role that OMPs play in virulence. The demonstration that modulation of expression of certain OMP genes might occur in B. avium has important implications for future research. Further characterization of the regulatory system of B. avium should be aided by its apparent similarity with the coordinate regulatory systems in the other members of the genus Bordetella.

LITERATURE CITED

1. Achtman, M., A. Mercer, B. Kusecek, A. Pohl, M. Heuzenroeder, W. Aaronson, A. Sutton, and R. P. Silver. 1983. Six widespread bacterial clones among Escherichia coli K1 isolates. *Infect. Immun.* 39:315-335.
2. Akeila, M. A, and Y. M. Saif. 1988. Protection of turkey poults from Bordetella avium infection and disease by pili and bacterins. *Avian Dis.* 32:641-649.
3. Arico, B., and R. Rappuoli. 1987. Bordetella parapertussis and Bordetella bronchiseptica contain transcriptionally silent pertussis toxin genes. *J. Bacteriol.* 169:2847-2853.
4. Arico, B., J. F. Miller, C. Roy, S. Stibitz, D. Monack, S. Falkow, R. Gross, and R. Rappuoli. 1989. Sequences required for expression of Bordetella pertussis virulence factors share homology with prokaryotic signal transduction proteins. *Proc. Natl. Acad. Sci. USA* 86:6671-6675.
5. Armstrong, S. K., and C. D. Parker. 1986. Heat-modifiable envelope proteins of Bordetella pertussis. *Infect. Immun.* 54:109-117.
6. Armstrong, S. K., and C. D. Parker. 1986. Surface proteins of Bordetella pertussis: Comparison of virulent and avirulent strains and effects of phenotypic modulation. *Infect. Immun.* 54:308-314.
7. Armstrong, S. K., T. R. Parr, Jr., C. D. Parker, and R. E. W. Hancock. 1986. Bordetella pertussis major outer membrane porin protein forms small, anion-selective channels in lipid bilayer membranes. *J. Bacteriol.* 166:212-216.
8. Arp, L. H. 1988. Bacterial infection of mucosal surfaces: An overview of cellular and molecular mechanisms. Pages 3-27 in J. A. Roth, ed. *Virulence mechanisms of bacterial pathogens*. American Society for Microbiology, Washington, DC.
9. Arp, L. H., and N. F. Cheville. 1984. Tracheal lesions in young turkeys infected with Bordetella avium. *Am. J. Vet. Res.* 45:2196-2200.
10. Arp, L. H., and J. A. Fagerland. 1987. Ultrastructural pathology of Bordetella avium infection in turkeys. *Vet. Pathol.* 24:411-418.

11. Arp, L. H., and D. H. Hellwig. 1988. Passive immunization versus adhesion of Bordetella avium to the tracheal mucosa of turkeys. Avian Dis. 32:494-500.
12. Arp, L. H., and S. M. McDonald. 1985. Influence of temperature on the growth of Bordetella avium in turkeys and in vitro. Avian Dis. 29:1066-1077.
13. Arp, L. H., D. H. Hellwig, and E. L. Huffman. 1988. Sialogangliosides as the putative tracheal mucosal receptors for Bordetella avium adhesins. Abstract B-164. 88th Ann. Meet. Am. Soc. Microbiol., Miami Beach.
14. Arp, L. H., R. D. Leyh, and R. W. Griffith. 1988. Adherence of Bordetella avium to tracheal mucosa of turkeys: Correlation with hemagglutination. Am. J. Vet. Res. 49:693-696.
15. Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff. 1981. Subtyping isolates of Haemophilus influenzae type b by outer membrane protein profiles. J. Infect. Dis. 143:668-676.
16. Barnes, H. J., and M. S. Hofstad. 1977. Turkey coryza: An emerging disease of young poults. J. Am. Vet. Med. Assoc. 171:1104.
17. Beher, M. G., C. A. Schnaitman, and A. P. Pugsley. 1980. Major heat-modifiable outer membrane protein in gram-negative bacteria: Comparison with the OmpA protein of Escherichia coli. J. Bacteriol. 143:906-913.
18. Benz, R. 1988. Structure and function of porins from gram-negative bacteria. Ann. Rev. Microbiol. 42:359-393.
19. Benz, R., A. Schmid, and R. E. W. Hancock. 1985. Ion selectivity of gram-negative bacterial porins. J. Bacteriol. 162:722-727.
20. Berkhoff, H. A., and G. D. Riddle. 1984. Differentiation of Alcaligenes-like bacteria of avian origin and comparison with Alcaligenes spp. reference strains. J. Clin. Microbiol. 19:477-481.
21. Berkhoff, H. A., H. J. Barnes, S. I. Ambrus, M. D. Kopp, G. D. Riddle, and D. C. Kradel. 1984. Prevalence of Alcaligenes faecalis in North Carolina broiler flocks and its relationship to respiratory disease. Avian Dis. 28:912-920.
22. Blackall, P. J., and J. G. Farrah. 1985. Isolation of Bordetella avium from poultry. Aust. Vet. J. 62:370-372.

23. Blake, M. S., and E. C. Gotschlich. 1984. Purification and partial characterization of the opacity-associated proteins of Neisseria gonorrhoeae. J. Exp. Med. 159:452-462.
24. Blake, M. S., L. M. Wetzler, E. C. Gotschlich, and P. A. Rice. 1989. Protein III: Structure, function, and genetics. Clin. Microbiol. Rev. 2:S60-S63.
25. Boucaud, J. -L., and R. Curtiss III. 1988. Mutagenesis of Bordetella avium genes involved in the adhesion of turkey tracheal cells. Abstract 382. 69th Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.
26. Boucard, J. -L., and C. Gentry-Weeks. 1989. Impaired adhesion of transposon Tnp_hoA-generated mutant of Bordetella avium. Abstract B-135. 89th Ann. Meet. Am. Soc. Microbiol., New Orleans.
27. Boycott, B. R., H. R. Wyman, and F. C. Wong. 1984. Alcaligenes faecalis rhinotracheitis in Manitoba turkeys. Avian Dis. 28:1110-1114.
28. Braun, V. 1975. Covalent lipoprotein from the outer membrane of Escherichia coli. Biochim. Biophys. Acta 415:335-377.
29. Brennan, M. J., Zhong, M. L., J. L. Cowell, M. E. Bisher, A. C. Steven, P. Novotny, and C. R. Manclark. 1988. Identification of a 69-kilodalton nonfimbrial protein as an agglutinin of Bordetella pertussis. Infect. Immun. 56:3189-3195.
30. Brown, M. R. W., and P. Williams. 1985. The influence of environment on envelope properties affecting survival of bacteria in infections. Ann. Rev. Microbiol. 39:527-556.
31. Cannon, J. G. 1989. Conserved lipoproteins of pathogenic Neisseria species bearing the H.8 epitope: Lipid-modified azurin and H.8 outer membrane protein. Clin. Microbiol. Rev. 2:S1-S4.
32. Charles, I. G., G. Dougan, D. Pickard, S. Chatfield, M. Smith, P. Novotny, P. Morrissey, and N. F. Fairweather. 1989. Molecular cloning and characterization of protective outer membrane protein P.69 from Bordetella pertussis. Proc. Natl. Acad. Sci. USA 86:3554-3558.
33. Chen, W., M. R. Alley, and B. W. Manktelow. 1988. Pneumonia in lambs inoculated with Bordetella parapertussis: Clinical and pathological studies. N. Z. Vet. J. 36:138-142.

34. Cookson, B. T., A. N. Tyler, and W. E. Goldman. 1988. Definitive structure of the peptidoglycan-derived tracheal cytotoxin of Bordetella pertussis. Abstract B-70. 88th Ann. Meet. Am. Soc. Microbiol., Miami Beach.
35. Cookson, B. T., H. -L. Cho, L. A. Herwaldt, and W. E. Goldman. 1989. Biological activities and chemical composition of purified tracheal cytotoxin of Bordetella pertussis. Infect. Immun. 57:2223-2229.
36. Corbeil, L. B., G. Chikami, M. Yarnell, J. Smith, and D. G. Guiney. 1988. Cloning and expression of genes encoding Haemophilus somnus antigens. Infect. Immun. 56:2736-2742.
37. De Ley, J., P. Segers, K. Kersters, W. Mannheim, and A. Lievens. 1986. Intra- and intergeneric similarities of the Bordetella ribosomal ribonucleic acid cistrons: Proposal for a new family, Alcaligenaceae. Int. J. Syst. Bacteriol. 36:405-414.
38. De Magistris, M. T., M. Romano, S. Nuti, R. Rappuoli, and A. Tagliabue. 1988. Dissecting human T cell responses against Bordetella species. J. Exp. Med. 168:1351-1362.
39. Duchene, M., C. Barron, A. Schweizer, B. -U. von Specht, and H. Domdey. 1989. Pseudomonas aeruginosa outer membrane lipoprotein I gene: Molecular cloning, sequence, and expression in Escherichia coli. J. Bacteriol. 171:4130-4137.
40. Ezzell, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark. 1981. Phase-shift markers in Bordetella: Alterations in envelope proteins. J. Infect. Dis. 143:562-569.
41. Ficken, M. D. 1983. Antibiotic aerosolization for treatment of alcaligenes rhinotracheitis. Avian Dis. 27:545-548.
42. Filion, R., S. Cloutier, E. R. Vrancken, and G. Bernier. 1967. Infection respiratoire du dindonneau causee par un microbe apparente au Bordetella bronchiseptica. Can. J. Comp. Vet. Sci. 31:129-134.
43. Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. 115:717-722.
44. Finlay, B. B., and S. Falkow. 1989. Common themes in microbial pathogenicity. Microbiol. Rev. 53:210-230.

45. Finlay, B. B., F. Heffron, and S. Falkow. 1989. Epithelial cell surfaces induce Salmonella proteins required for bacterial adherence and invasion. Science 243:940-943.
46. Gentry-Weeks, C. R., and R. Curtiss III. 1988. Molecular cloning of the dermonecrotic toxin gene of Bordetella avium. Abstract 381. 69th Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.
47. Gentry-Weeks, C., and D. L. Provence. 1990. Isolation and characterization of phase variants of Bordetella avium. Abstract B-147. 90th Ann. Meet. Am. Soc. Microbiol., Anaheim.
48. Gentry-Weeks, C., A. -L. Hultsch, S. M. Kelly, and R. Curtiss III. 1990. Molecular cloning of outer membrane protein genes of Bordetella avium 197 and expression of a B. avium 21-kDa outer membrane protein in Salmonella typhimurium. Abstract B-148. 90th Ann. Meet. Am. Soc. Microbiol., Anaheim.
49. Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss III. 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of Bordetella avium. Infect. Immun. 56:1698-1707.
50. Goldman, W. E. 1986. Bordetella pertussis tracheal cytotoxin: Damage to the respiratory epithelium. Pages 65-69 in L. Leive, ed. Microbiology-1986. American Society for Microbiology, Washington, DC.
51. Goldman, W. E., D. G. Klapper, and J. B. Baseman. 1982. Detection, isolation, and analysis of a released Bordetella pertussis product toxic to cultured tracheal cells. Infect. Immun. 36:782-794.
52. Gomez-Miguel, M. J., K. Moriyon, and J. Lopez. 1987. Brucella outer membrane lipoprotein shares antigenic determinants with Escherichia coli Braun lipoprotein and is exposed on the cell surface. Infect. Immun. 55:258-262.
53. Gould-Kostka, J. L., D. L. Burns, M. J. Brennan, and C. R. Manclark. 1990. Purification and analysis of the antigenicity of a 69000 Da protein from Bordetella pertussis. FEMS Microbiol. Let. 67:285-290.
54. Gray, J. G., J. F. Roberts, and D. G. Simmons. 1983. In vitro cytotoxicity of an Alcaligenes faecalis and its relationship in vivo tracheal pathologic changes in turkeys. Avian Dis. 27:1142-1150.

55. Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons. 1981. Cytotoxic activity of pathogenic Alcaligenes faecalis in turkey tracheal organ cultures. Am. J. Vet. Res. 42:2184-2186.
56. Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons. 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an Alcaligenes faecalis isolate. Infect. Immun. 42:350-355.
57. Griffiths, E., H. Chart, and P. Stevenson. 1988. High-affinity iron uptake systems and bacterial virulence. Pages 121-137 in J. A. Roth, ed. Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, DC.
58. Gross, R., and R. Rappuoli. 1988. Positive regulation of pertussis toxin expression. Proc. Natl. Acad. Sci. USA 85:3913-3917.
59. Heller, E. D., Y. Weisman, and A. Aharonovovitch. 1984. Experimental studies on turkey coryza. Avian Pathol. 13:137-143.
60. Hellwig, D. H., and L. H. Arp. 1990. Identification of Bordetella avium antigens recognized after experimental inoculation in turkeys. Am. J. Vet. Res. 51:1188-1191.
61. Hellwig, D. H., L. H. Arp, and J. A. Fagerland. 1988. A comparison of outer membrane proteins and surface characteristics of adhesive and non-adhesive phenotypes of Bordetella avium. Avian Dis. 32:787-792.
62. Hewlett, E. L. 1990. Chap. 208. Bordetella species. Pages 1756-1762 in G. L. Mendell, R. G. Douglas, and J. E. Bennett, eds. Principles and practice of infectious diseases. 3rd Ed. Churchill Livingstone, New York.
63. Hinz, K. -H. 1986. Avian bordetellosis-a short review. Isr. J. Vet. Med. 42:141-145.
64. Hinz, K. -H., G. Glunder, and H. Luders. 1978. Acute respiratory disease in turkey poults caused by Bordetella bronchiseptica-like bacteria. Vet. Rec. 103:262-263.
65. Hinz, K. -H., G. Glunder, and K. J. Romer. 1983. A comparative study of avian Bordetella-like strains, Bordetella bronchiseptica, Alcaligenes faecalis and other related nonfermentatable bacteria. Avian Pathol. 12:263-276.

66. Hogg, N. 1989. The leukocyte integrins. *Immunol. Today* 10:111-114.
67. Ishikawa, H., and Y. Isayama. 1987. Evidence for sialyl glycoconjugates as receptors for Bordetella bronchiseptica on swine nasal mucosa. *Infect. Immun.* 55:1607-1609.
68. Ishikawa, H., and Y. Isayama. 1987. Effect of antigenic modulation and phase variation on adherence of Bordetella bronchiseptica to porcine nasal epithelial cells. *Am. J. Vet. Res.* 48:1689-1691.
69. Jackwood, M. W., and Y. M. Saif. 1987. Pili of Bordetella avium: Expression, characterization, and role in in vitro adherence. *Avian Dis.* 31:277-286.
70. Jackwood, M. W., D. A. Hilt, and P. A. Dunn. 1990. Colonial phenotypic variation in Bordetella avium. Abstract P18. 71st Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.
71. Jackwood, M. W., Y. M. Saif, and D. L. Coplin. 1987. Isolation and characterization of Bordetella avium plasmids. *Avian Dis.* 31:782-786.
72. Jackwood, M. W., M. Sasser, and Y. M. Saif. 1986. Contribution to the taxonomy of the turkey coryza agent: Cellular fatty acid analysis of the bacterium. *Avian Dis.* 30:172-178.
73. Jackwood, M. W., Y. M. Saif, P. D. Moorhead, and R. N. Dearth. 1985. Further characterization of the agent causing coryza in turkeys. *Avian Dis.* 29:690-705.
74. Johnston, K. H., K. K. Holmes, and E. C. Gotschlich. 1976. The serological classification of Neisseria gonorrhoeae. I. Isolation of the outer membrane complex responsible for serotypic specificity. *J. Exp. Med.* 143:741-758.
75. Kadner, R. J. 1978. Repression of the synthesis of the vitamin B₁₂ receptor in Escherichia coli. *J. Bacteriol.* 136:1050-1057.
76. Kelly, B. J., G. Y. Ghazikhanian, and B. Mayeda. 1986. Clinical outbreak of Bordetella avium infection in two turkey breeder flocks. *Avian Dis.* 30:234-237.
77. Kersters, K., K. H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley. 1984. Bordetella avium sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int. J. Syst. Bacteriol.* 34:56-70.

78. Kessel, M., M. J. Brennan, B. L. Trus, M. E. Bisher, and A. C. Steven. 1988. Naturally crystalline porin in the outer membrane of Bordetella pertussis. J. Mol. Biol. 203:275-278.
79. Knapp, S., and J. J. Mekalonos. 1988. Two trans-acting regulatory genes (vir and mod) control antigenic modulation in Bordetella pertussis. J. Bacteriol. 170:5059-5066.
80. Kobisch, M., and P. Novotny. 1990. Identification of a 68-kilodalton outer membrane protein as the major protective antigen of Bordetella bronchiseptica by using specific-pathogen-free piglets. Infect. Immun. 58:352-357.
81. Kofoed, E. C., and J. S. Parkinson. 1988. Transmitter and receiver modules in bacterial signaling proteins. Proc. Natl. Acad. Sci. USA. 85:4981-4985.
82. Lacey, B. W. 1960. Antigenic modulation of Bordetella pertussis. J. Hyg. 58:57-93.
83. Lanning, S. R., D. A. Freeman, and G. H. Luginbuhl. 1989. Lipopolysaccharide demonstrated to have a significant role in biologic activity of heat stable B. avium toxin. Abstract B-204. 89th Ann. Meet. Am. Soc. Microbiol., New Orleans.
84. Laoide, B. M., and A. Ullman. 1990. Virulence dependent and independent regulation of the Bordetella pertussis cya operon. EMBO J. 9:999-1005.
85. Lax, A. J. 1985. Is phase variation in Bordetella caused by mutation and selection? J. Gen. Microbiol. 131:913-917.
86. Leininger, E., J. G. Kenimer, C. A. Ewanowich, M. S. Peppler, and M. J. Brennan. 1990. Role of the RGD sequences of Bordetella pertussis adhesins. Abstract B-138. 90th Ann. Meet. Am. Soc. Microbiol., Anaheim.
87. Leininger-Zapata, E., M. J. Brennan, J. G. Kenimer, I. Charles, N. Fairweather, and P. Novotny. 1989. Cell attachment to putative adhesins from Bordetella pertussis. Abstract B-123. 89th Ann. Meet. Am. Soc. Microbiol., New Orleans.
88. Leyh, R. D., and R. W. Griffith. 1989. Outer membrane protein profiles of Bordetella avium. Abstract 69. 70th Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.
89. Leyh, R. D., R. W. Griffith, and L. H. Arp. 1988. Transposon mutagenesis in Bordetella avium. Am. J. Vet. Res. 49:687-692.

90. Lugtenburg, B., and L. van Alphen. 1983. Molecular architecture and functioning of the outer membrane of Escherichia coli and other gram-negative bacteria. Biochim. Biophys. Acta 737:51-115.
91. Marshall, D. R., D. G. Simmons, and J. G. Gray. 1984. Evidence for adherence-dependent cytotoxicity of Alcaligenes faecalis in turkey tracheal organ cultures. Avian Dis. 28:1007-1015.
92. Marshall, D. R., D. G. Simmons, and J. G. Gray. 1985. An Alcaligenes faecalis isolate from turkeys: Pathogenicity in selected avian and mammalian species. Am. J. Vet. Res. 46:1181-1184.
93. Miller, J. F., J. J. Mekalanos, and S. Falkow. 1989. Coordinate regulation and sensory transduction in the control of bacterial virulence. Science 243:916-922.
94. Mizuno, T., and M. Kageyama. 1979. Isolation and characterization of a major outer membrane protein of Pseudomonas aeruginosa. J. Biochem. 85:115-122.
95. Monack, D. M., B. Arico, R. Rappuoli, and S. Falkow. 1989. Phase variants of Bordetella bronchiseptica arise by spontaneous deletions in the vir locus. Mol. Microbiol. 3:1719-1728.
96. Montaraz, J. A., P. Novotny, and J. Ivanyi. 1985. Identification of a 68-kilodalton protective protein antigen from Bordetella bronchiseptica. Infect. Immun. 47:744-751.
97. Mooi, F. R. 1988. Virulence factors of Bordetella pertussis. Antonie Van Leeuwenhoek 54:465-474.
98. Mooi, F. R., H. G. J. van der Heide, A. R. ter Avest, K. G. Welinder, I. Livey, B. A. M. van der Zeijst, and W. Gaastra. 1987. Characterization of fimbrial subunits from Bordetella species. Microbial Pathogen. 2:473-484.
99. Murphy, T. F., and L. C. Bartos. 1989. Surface-exposed and antigenically conserved determinants of outer membrane proteins of Branhamella catarrhalis. Infect. Immun. 57:2938-2941.
100. Musser, J. M., E. L. Hewlett, M. S. Peppler, and R. K. Selander. 1986. Genetic diversity and relationships in populations of Bordetella spp. J. Bacteriol. 166:230-237.
101. Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. 49:1-32.

102. Novotny, P., A. P. Chubb, K. Cownley, and J. A. Montaraz. 1985. Adenylate cyclase activity of a 68,000-molecular-weight protein isolated from the outer membrane of Bordetella bronchiseptica. Infect. Immun. 50:199-206.
103. Novotny, P., M. Kobisch, K. Cownley, A. P. Chubb, and J. A. Montaraz. 1985. Evaluation of Bordetella bronchiseptica vaccines in specific-pathogen-free piglets with bacterial cell surface antigens in enzyme-linked immunosorbent assay. Infect. Immun. 50:190-198.
104. Ofek, I. and E. H. Beachey. 1980. Bacterial adherence. Adv. Intern. Med. 25:503-532.
105. Osborn, M. J., and H. C. P. Wu. 1980. Proteins of the outer membrane of gram-negative bacteria. Ann. Rev. Microbiol. 34:369-422.
106. Parker, C. D., and S. K. Armstrong. 1988. Surface proteins of Bordetella pertussis. Rev. Infect. Dis. 10:S327-S330.
107. Parker, C. D., L. V. Branes, S. K. Armstrong, D. W. Frank, and A. Cole. 1985. Cell surface antigens of Bordetella pertussis. Dev. Biol. Stand. 61:123-136.
108. Parton, R., and A. C. Wardlaw. 1975. Cell-envelope proteins of Bordetella pertussis. J. Med. Microbiol. 8:47-57.
109. Peppler, M. S., and M. E. Schrupf. 1984. Phenotypic variation and modulation in Bordetella bronchiseptica. Infect. Immun. 44:681-687.
110. Rapp, V. J., R. S. Munson, Jr., and R. F. Ross. 1986. Outer membrane protein profiles of Haemophilus pleuropneumoniae. Infect. Immun. 52:414-420.
111. Redhead, K. 1983. Variability in the surface exposure of the outer membrane proteins of Bordetella pertussis. FEMS Microbiol. Lett. 17:35-39.
112. Relman, D., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. D. Wright. 1990. Recognition of a bacterial adhesin by an integrin: Macrophage CR3 ($\alpha_{M} \beta_2$, Cd11b/CD18) binds filamentous hemagglutinin of Bordetella pertussis. Cell 61:1375-1382.
113. Rhoades, K. R., and R. B. Rimler. 1987. The effects of heat-labile Bordetella avium toxin on turkey poults. Avian Dis. 31:345-350.

114. Rimler, R. B. 1985. Turkey coryza: Toxin production by Bordetella avium. Avian Dis. 29:1043-1047.
115. Rimler, R. B., and K. R. Rhoades. 1986. Turkey coryza: Selected tests for detection and neutralization of Bordetella avium heat-labile toxin. Avian Dis. 30:808-812.
116. Rimler, R. B., and D. G. Simmons. 1983. Differentiation among bacteria isolated from turkeys with coryza (rhinotracheitis). Avian Dis. 27:491-500.
117. Robinson, A., and D. C. Hawkins. 1983. Structure and biological properties of solubilized envelope proteins of Bordetella pertussis. Infect. Immun. 39:590-598.
118. Roy, C. R., J. F. Miller, and S. Falkow. 1989. The bvgA gene of Bordetella pertussis encodes a transcriptional activator required for coordinate regulation of several virulence genes. J. Bacteriol. 171:6338-6344.
119. Saif, Y. M., P. D. Moorhead, and R. E. Whitmoyer. 1981. Scanning electron microscopy of tracheas from turkey poultts infected with Alcaligenes faecalis. Avian Dis. 25:730-735.
120. Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. J. Jackwood. 1980. Observations on Alcaligenes faecalis infection in turkeys. Avian Dis. 24:655-684.
121. Scarlato, V., A. Prugnola, B. Arico, and R. Rappuoli. 1990. Positive transcriptional feedback at the bvg locus controls expression of virulence factors in Bordetella pertussis. Proc. Natl. Acad. Sci. USA 87:6753-6757.
122. Schnaitman, C. A. 1970. Protein composition of the cell wall and cytoplasmic membrane of Escherichia coli. J. Bacteriol. 104:890-901.
123. Schnaitman, C. A. 1971. Solubilization of the cytoplasmic membrane of Escherichia coli by Triton X-100. J. Bacteriol. 108:545-552.
124. Shahin, R. D., M. J. Brennan, Z. M. Li, B. D. Meade, and C. R. Manclark. 1990. Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of Bordetella pertussis. J. Exp. Med. 171:63-73.
125. Simmons, D. G., and J. G. Gray. 1979. Transmission of acute respiratory disease (rhinotracheitis) of turkeys. Avian Dis. 23:132-138.

126. Simmons, D. G., C. Dees, and L. P. Rose. 1986. A heat-stable toxin isolated from the turkey coryza agent Bordetella avium. Avian Dis. 30:761-765.
127. Simmons, D. G., L. P. Rose, and J. G. Gray. 1980. Some physical, biochemic, and pathologic properties of Alcaligenes faecalis, the bacterium causing rhinotracheitis (coryza) in turkey poults. Avian Dis. 24:82-90.
128. Simmons, D. G., L. P. Rose, K. A. Brogden, and R. B. Rimler. 1984. Partial characterization of the hemagglutinin of Alcaligenes faecalis. Avian Dis. 28:700-709.
129. Simmons, D. G., L. P. Rose, F. M. McCorkle, and G. H. Luginbuhl. 1983. Histamine-sensitizing factor of Alcaligenes faecalis. Avian Dis. 27:171-177.
130. Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller. 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. 23:194-203.
131. Simmons, D. G., L. P. Rose, F. J. Fuller, L. C. Maurer, and G. H. Luginbuhl. 1986. Turkey coryza: Lack of correlation between plasmids and pathogenicity of Bordetella avium. Avian Dis. 30:593-597.
132. Slavik, M. F., J. K. Skeeles, J. N. Beasley, G. C. Harris, P. Roblee, and D. Hellwig. 1981. Effect of humidity on infection of turkeys with Alcaligenes faecalis. Avian Dis. 25:936-942.
133. Stibitz, S., A. A. Weiss, and S. Falkow. 1988. Genetic analysis of a region of the Bordetella pertussis chromosome encoding filamentous hemagglutinin and the pleiotropic regulatory locus vir. J. Bacteriol. 170:2904-2913.
134. Stibitz, S., W. Aaronson, D. Monack, and S. Falkow. 1989. Phase variation in Bordetella pertussis by frameshift mutation in a gene for a novel two-component system. Nature 338:266-269.
135. Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. 53:450-490.
136. Tabatabai, L. B., B. L. Deyoe, and A. E. Ritchie. 1979. Isolation and characterization of toxic fractions from Brucella abortus. Infect. Immun. 26:668-679.

137. Thomas, M. G., K. Redhead, and H. P. Lambert. 1989. Human serum antibody responses to Bordetella pertussis infection and pertussis vaccination. J. Infect. Dis. 159:211-218.
138. Tuomanen, E. 1988. Bordetella pertussis adhesins. Pages 75-94 in A. C. Wardlaw, and R. Parton, eds. Pathogenesis and immunity in pertussis. John Wiley and Sons Ltd., Sussex.
139. Tuomanen, E., and A. Weiss. 1985. Characterization of two adhesins of Bordetella pertussis for human ciliated respiratory-epithelial cells. J. Infect. Dis. 152:118-125.
140. Tuomanen, E. I., J. Nedelman, J. O. Hendley, and E. L. Hewlett. 1983. Species specificity of Bordetella adherence to human and animal ciliated respiratory epithelial cells. Infect. Immun. 42:692-695.
141. Tuomanen, E., H. Towbin, G. Rosenfelder, D. Braun, G. Larson, G. C. Hansson, and R. Hill. 1988. Receptor analogs and monoclonal antibodies that inhibit adherence of Bordetella pertussis to human ciliated respiratory epithelial cells. J. Exp. Med. 168:267-277.
142. Van Alstine, W. G., and L. H. Arp. 1987. Effects of Bordetella avium toxin on turkey tracheal organ cultures as measured with a tetrazolium-reduction assay. Avian Dis. 31:136-139.
143. Van Alstine, W. G., and L. H. Arp. 1987. Influence of Bordetella avium infection on association of Escherichia coli with turkey trachea. Am. J. Vet. Res. 48:1574-1576.
144. Van Alstine, W. G., and L. H. Arp. 1987. Effects of Bordetella avium infection on the pulmonary clearance of Escherichia coli in turkeys. Am. J. Vet. Res. 48:922-926.
145. Van Alstine, W. G., and L. H. Arp. 1988. Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with Bordetella avium. Am. J. Vet. Res. 49:835-839.
146. Verstrete, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter. 1982. Outer membrane proteins of Brucella abortus: Isolation and characterization. Infect. Immun. 35:979-989.
147. Wardlaw, A. C., and R. Parton. 1983. Bordetella pertussis toxins. Pharmacol. Ther. 19:1-53.

148. Weiss, A. A., and S. Falkow. 1984. Genetic analysis of phase change in Bordetella pertussis. Infect. Immun. 43:263-269.
149. Weiss, A. A., and E. L. Hewlett. 1986. Virulence factors of Bordetella pertussis. Ann. Rev. Microbiol. 40:661-686.
150. Weiss, A. A., A. R. Melton, K. E. Walker, C. Andraos-Selim, and J. J. Meidl. 1989. Use of the promoter fusion transposon Tn5 lac to identify mutations in Bordetella pertussis vir-regulated genes. Infect. Immun. 57:2674-2682.
151. Wensink, J. and B. Witholt. 1981. Outer-membrane vesicles released by normally growing Escherichia coli contain very little lipoprotein. Eur. J. Biochem. 116:331-335.
152. Yersin, A. G., F. W. Edens, D. G. Simmons, and D. J. Meuten. 1987. Alterations of thymic architecture by Bordetella avium toxin. Abstract 244. 68th Ann. Meet. Conf. Res. Work. Anim. Dis., Chicago.

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